IDENTIFICATION OF PEPTIDE SUBSTRATES OF CALCIUM-DEPENDENT PROTEIN KINASE FROM RANDOM PEPTIDE PHAGE DISPLAY LIBRARIES AND PHOSPHORYLATION STUDIES OF THE PEPTIDE SUBSTRATE IN TRANSGENIC TOBACCO CELLS

By

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In an effort to identify a peptide inhibitor and define the substrate preference of soybean calcium-dependent protein kinase (CDPK), a 15-residue random peptide phage display library was biopanned with purified $CDPK_{\alpha}$ (one CDPK isoform from soybean). After three rounds of biopanning, 43 clones were selected at random for DNA sequencing. The specific binding between the individual phage and $CDPK_{\alpha}$ was confirmed by an independent binding assay. One peptide corresponding to the insert sequence of the specific binding phage 6-3 plus the surrounding amino acid residues was synthesized and shown to be a substrate of CDPK with low K_M. A new random peptide phage display library

was constructed by adding 10 random amino acid residues to the amino-terminal side of the fixed peptide 6-3 sequence (RHPTLTRSPTLRNIQ). The new library was screened, and sequence analysis of the randomly selected 10 clones showed the enrichment of peptides containing hydrophobic residues such as aromatic amino acids and/or aliphatic amino acids. With the additional 10 residues at the amino-terminal side of peptide 6-3, the 25-residue peptides displayed by the phage were still phosphorylated by CDPK. Sequence 7-8,11 (VSPRSFWTTW) appeared twice in the sequence analysis and agrees well with the consensus alignment. The sequences of peptide 7-8,11 and 6-3 were selected and fused to a double hemagglutinin epitope tag, and cloned into a plant expression vector pBI121. The constructed expression vector pBI121-phag was transformed into tobacco by *Agrobacterium*-mediated transformation. The transformed tobacco suspension culture was established. The tobacco-expressed peptide substrate was phosphorylated by recombinant $CDPK_{\alpha}$ and endogenous calcium-dependent protein kinase in the tobacco cell extract. Metabolic labeling of transgenic tobacco cells showed increased phosphorylation of the peptide substrate in response to hydrogen peroxide treatment.

CHAPTER 1
LITERATURE REVIEW

Introduction

Calcium plays an important role as a second messenger by regulating various aspects of cellular signal transduction in plants and animals. The signal-induced changes in intracellular free Ca^{2+} concentration have been portrayed as a switch turning on a variety of cellular processes. In plants, various external stimuli (light, wind, and gravity), phytohormones (auxin, gibberellic acid, abscisic acid, ethylene, and cytokinin), and environmental stresses (cold shock, drought, mechanical stimulation, hypoosmotic shock, pathogen elicitors) can cause transient changes in cytoplasmic free calcium concentration. The connection of calcium to the signal transduction pathways in plants has been extensively reviewed (Bush, 1993; Bush, 1995; Gilroy et al., 1993; Gilroy and Trewavas, 1994; Poovaiah and Reddy, 1993; Trewavas and Gilroy, 1991; Trewavas and Knight, 1994).

Protein phosphorylation is one of the major mechanisms by which eukaryotic cells transduce extracellular signals to intracellular responses (Cohen, 1992; Edelman et al., 1987). Calcium-regulated protein kinases are involved in amplifying and diversifying the action of Ca^{2+}-mediated responses. Biochemical and molecular evidence indicates that calcium-dependent, calmodulin-

independent protein kinases (CDPK) are the predominant calcium-regulated protein kinases in plants. However, homologues of protein kinase C or Ca^{2+}/camodulin-dependent protein kinase (Lu et al., 1996; Satterlee and Sussman, 1998; Watillon et al., 1992; Watillon et al., 1993) have been identified in plants. CDPKs are a family of enzymes that have similar primary structures consisting of a catalytic domain, an autoinhibitory domain, and a calmodulin-like domain (Roberts and Harmon, 1992). DNA encoding CDPKs have been cloned from soybean and other plants such as carrot, *Arabidopsis*, rice, corn, mungbean, and also from algae and protists. While numerous CDPK genes have been identified, an understanding of their physiological roles remains elusive.

Calcium-Dependent Protein Kinases

Molecular and Biochemical Evidence of the CDPK Family

CDPK cloning

The plant calcium-dependent protein kinase was first purified to near homogeneity from soybean cell suspension cultures (Putnam-Evans et al., 1990). The kinase itself binds calcium with high affinity. The kinase activity is activated by the direct binding of calcium and requires neither calmodulin nor phospholipids or diacylglycerol. This led to the first cloning of a cDNA of CDPK from soybean by Harper et al. (1991). The protein encoded by this cDNA was named $CDPK_{\alpha}$. $CDPK_{\alpha}$ contains a catalytic domain similar to that of calmodulin-dependent protein kinases and a calmodulin-like domain with four calcium-binding sites (EF hands). Since then, a large number of CDPK genes have been

cloned from soybean (Lee et al., 1997) and other plants, including carrot (Suen and Choi, 1991), rice (Breviario et al., 1995; Kawasaki et al., 1993), *Arabidopsis* (Harper et al., 1993; Hong et al., 1996; Hrabak et al., 1996; Tahtiharju et al., 1997; Urao et al., 1994a; Urao et al., 1994b), maize (Berberich and Kusano, 1997; Estruch et al., 1994; Saijo et al., 1997; Takazawa et al., 1996), alfalfa (Monroy and Dhindsa, 1995), and mungbean (Botella et al., 1996). CDPK genes have also been cloned from protists such as *Chlamydomonas* (Siderius et al., 1997), *Plasmodium falciparum* (Farber et al., 1997; Zhao et al., 1993), *Eimeria* (Dunn et al., 1996), and *Paramecium* (Kim et al., 1998). Data of CDPK cDNA and genomic DNA cloning indicate that there are multiple CDPK isoforms in plants. Southern blot analysis of genomic DNA from soybean (Harper et al., 1991), *Arabidopsis* (Harper et al., 1993; Hong et al., 1996; Urao et al., 1994b), and mungbean (Botella et al., 1996) provides further evidence for the presence of a CDPK multigene family in plants.

Induction of CDPK expression

CDPK genes encode proteins with a Ser/Thr protein kinase catalytic domain, a junction domain, and a calcium-binding domain with four EF-hands. As the structure implies, the activity of CDPK is directly activated by calcium. This indicates that CDPKs may be involved in signal transduction pathways mediated by calcium. Interestingly, expression of two CDPK genes is tissue specific (Estruch et al., 1994; Kawasaki et al., 1993). Expression of one maize CDPK is pollen specific, and its transcription is restricted to the late stages of pollen development (Estruch et al., 1994). A rice CDPK (SPK) is specifically expressed in developing seeds, and its expression pattern is very similar to those of genes encoding starch-synthesizing enzymes, seed lipid-synthesizing

enzymes, as well as genes encoding seed storage proteins (Kawasaki et al., 1993). Some mRNAs encoding CDPK have been reported to be inducible by physiological stimuli and environmental stresses (Berberich and Kusano, 1997; Botella et al., 1996; Breviario et al., 1995; Monroy and Dhindsa, 1995; Urao et al., 1994b). Two CDPK genes, AtCDPK1 and AtCDPK2, isolated from a cDNA library prepared from dehydrated *Arabidopsis thaliana*, are rapidly induced by drought and high-salt stress but not by exogenous ABA (abscisic acid) treatment, low-temperature stress, or heat stress (Urao et al., 1994b). These findings suggest that a change in the osmotic potential of the environment can serve as a trigger for the induction of AtCDPK1 and AtCDPK2. Low temperature-induced CDPKs have been cloned in alfalfa (Monroy and Dhindsa, 1995), *Arabidopsis* (Tahtiharju et al., 1997), and maize (Berberich and Kusano, 1997). The transcript level of one of two CDPKs from alfalfa was markedly upregulated at 4 °C (Monroy and Dhindsa, 1995). Using restriction fragment length polymorphism-coupled domain-directed differential display, five CDPK clones were identified that showed differential regulation by cold (Tahtiharju et al., 1997). The low-temperature induction of ZmCDPK1 was increased by cycloheximide treatment along with elevation of the transcript level of another cold-inducible gene mlip15 (encoding a DNA-binding protein of the basic region/leucine zipper type) and Adh1 (alcohol dehydrogenase 1). VrCDPK1 was cloned from an IAA (indole-3-acetic acid)-induced mungbean cDNA library. The transcription of VrCDPK is induced by mechanical strain, salt stress, and IAA treatment (Botella et al., 1996). OsCPK2, one of the rice CDPK genes, was isolated from a cDNA library constructed from rice coleoptiles. The amount of OsCPK2 transcript declines by anoxic treatment and white light treatment of the coleoptiles (Breviario et al.,

1995). A study using maize leaf protoplasts with transient expressed constitutively active CDPK isoforms showed that AtCDPK1 (Urao et al., 1994b) activates a stress-inducible (cold, salt, dark, and ABA treatment-inducible) promoter in the absence of environmental stresses (Sheen, 1996). Whereas AtCDPK itself is inducible by drought and salt, it is intriguing to know if plants perceive the stimuli and response to signals in a linear fashion or by multiple signal transduction pathways (Harmon, 1997).

Purification of CDPK from plants

The presence and biochemical characterization of CDPK in plants and protists was reviewed extensively in 1992 (Roberts and Harmon, 1992). Since then, more CDPKs have been completely or partially purified from plants including zucchini (Verhey et al., 1993), groundnut (DasGupta, 1994), tobacco (Kunz et al., 1996; Ohto and Nakamura, 1995), rice (Abo-el-Saad and Wu, 1995; Karibe et al., 1996), winged bean (Ganguly and Singh, 1998; Saha and Singh, 1995), potato (MacIntosh et al., 1996), maize and sorghum (Pestenacz and Erdei, 1996), and mango (Frylinck and Dubery, 1998), and from protists including the green alga *Dunaliella tertiolecta* (Yuasa and Muto, 1992) and the protozoan *Paramecium* (Son et al., 1993).

Induction of CDPK activity

The activity of several CDPKs can be induced. In rice seeds, a plasma membrane-associated calcium-dependent protein kinase is induced by gibberellin (Abo-el-Saad and Wu, 1995). Sugar induces the increased activity of

calcium-dependent protein kinases associated with the plasma membrane in leaf tissues of tobacco (Ohto and Nakamura, 1995), and calcium-dependent protein kinase in maize and sorghum is induced by polyethylene glycol (Pestenacz and Erdei, 1996). The activity of CDPK is detected in certain developmental stages in potato tuberization (MacIntosh et al., 1996). CDPK activities are present in different organs such as pollen tubes of *Nicotiana alata* (Kunz et al., 1996), seeds of groundnut (DasGupta, 1994), and fruits of mango (Frylinck and Dubery, 1998). Western blotting analysis using polyclonal antibodies of atCDPK6 and atCDPK9 showed that *Arabidopsis* CDPKs are present in different organs such as flower, leaf, stem, root, and pod (Hong et al., 1996).

Structural Properties and Mechanism of CDPK

CDPKs are a family of enzymes with similar primary structures. From amino terminus to carboxyl terminus, CDPK contains a catalytic domain, a junction domain, and a calmodulin-like domain (Roberts and Harmon, 1992). The amino acid sequence identity between CDPK isoforms ranges from 50% to 95%. CDPK was first cloned from soybean (Harper et al., 1991). The amino terminal catalytic domain of soybean $CDPK_\alpha$ is closely related in sequence to that of calcium/calmodulin-dependent protein kinase from the rat brain. The carboxyl terminal regulatory domain has four putative helix-loop-helix or EF-hand calcium-binding sites and is 39% identical to spinach calmodulin. The short junction region between the kinase and calmodulin-like domains functions as an autoinhibitory domain (Harmon et al., 1994; Harper et al., 1994; Huang et al., 1996; Yoo and Harmon, 1996).

Catalytic domain

The catalytic domain of CDPK, most closely related in sequence to that of Ca^{2+}/camodulin-dependent protein kinases, possesses twelve conservative subdomains in the protein kinase superfamily (Hanks and Hunter, 1995; Hanks and Quinn, 1991; Hanks et al., 1988). Twelve kinase subdomains are recognized as being invariant or nearly invariant throughout the kinase superfamily and hence strongly implicated as playing essential roles in enzyme function. Although the amino terminal region of CDPKs varies in length and primary sequence, the catalytic core sequence of 250-300 amino acid residues are identified in all CDPKs, and the characteristic ten invariant residues for the Ser/Thr protein kinase subfamily are conserved.

The crystal structures of a number of protein kinases were resolved in the past few years (De Bondt et al., 1993; Goldberg et al., 1996; Hu et al., 1994; Hubbard et al., 1994; Knighton et al., 1991a; Knighton et al., 1991b; Longenecker et al., 1996; Owen et al., 1995; Zhang et al., 1994). The three-dimensional structures of reported protein kinases are very similar . They all consist of one amino terminal small lobe and one carboxyl terminal large lobe with a deep cleft in between, which constitutes the active site of the enzyme where most of the conserved residues lie. Since all the CDPK catalytic domains contain the conserved residues for the Ser/Thr protein kinase family, the kinase domain of CDPKs likely adopts the same three-dimensional structure of the reported kinases. The crystal structures of protein kinases provide the structural basis by which to correlate the features discerned from sequence alignments with their

catalytic function (Johnson et al., 1996; Taylor et al., 1993a; Taylor et al., 1993b; Wei et al., 1994).

Junction domain

The junction domain of CDPK contains the autoinhibitory domain and calmodulin-binding domain (Harmon et al., 1994; Harper et al., 1994), and it is highly conserved throughout the CDPK family. The junction domain of soybean $CDPK_\alpha$ has some identity with the autoinhibitory domain of calmodulin-dependent protein kinase type II (Harper et al., 1991). Biochemical studies on protein kinases such as cAMP-dependent protein kinase, calmodulin-dependent protein kinase, myosin light chain kinase, protein kinase C, and protein phosphatases such as calcineurin and protein phosphatase-1 indicate that the autoinhibitory domain acts as a pseudosubstrate which blocks the enzyme active site to render the enzyme inactive (Kemp et al., 1994; Kemp and Pearson, 1991; Kemp et al., 1991). The crystal structure of the kinase domain and regulatory region of the large muscle protein twitchin showed that a 60-residue segment immediately following the catalytic domain of the enzyme binds to and blocks the active site, consistent with the pseudosubstrate hypothesis (Hu et al., 1994). The similar autoinhibition mechanism was demonstrated in soybean (Harmon et al., 1994) and *Arabidopsis* (Harper et al., 1994; Hong et al., 1996) CDPKs. Synthetic peptides corresponding to the pseudosubstrate site of the junction domain act as competitive inhibitors of CDPK (Harmon et al., 1994; Hong et al., 1996). Removal of the autoinhibitory domain and the calmodulin-like domain results in a constitutively active calcium-independent protein kinase (Harmon et al., 1994; Harper et al., 1994).

The junction domain also contains the calmodulin-binding region, which is located at the carboxyl side of the pseudosubstrate site and partially overlaps the pseudosubstrate site (Harmon et al., 1994). The binding of the calmodulin-like domain to the junction domain in the presence of calcium disrupts the intramolecular binding of the pseudosubstrate region with the enzyme active site and leads to the activation of the CDPK by calcium (Huang et al., 1996; Yoo and Harmon, 1996). Kinetic binding analyses determined a dissociation constant (K_d) of 6×10^{-6} M between the calmodulin-like domain (CLD) and the junction domain. The activity of *Arabidopsis* CDPK lacking CLD can be activated by exogenous calmodulin or an isolated CLD in the presence of calcium (Huang et al., 1996). For soybean $CDPK_\alpha$, a mutant lacking all the CLD can be activated only partially by the addition of calcium and CLD. Another CDPK mutant with half of the CLD retained in its sequence can be fully activated by the addition of CLD but poorly by calmodulin (Yoo and Harmon, 1996). Studies on the activation mechanism of soybean and *Arabidopsis* CDPKs all support the idea that binding of the calmodulin-like domain to the junction domain contributes to the activation of CDPK. However, the data of Yoo and Harmon (1996) indicate that the binding of the CLD and the junction domain alone is not sufficient for the complete activation of CDPK.

Calmodulin-like domain

The carboxyl terminal domain of CDPK resembles the calcium-binding protein, calmodulin, and so was named the calmodulin-like domain (Roberts and Harmon, 1992). CLD contains four putative EF-hands with 39% amino acid sequence identity to calmodulin from spinach (Harper et al., 1991). Each of the

four EF-hand motifs of CDPK contains all the requirements for a Ca^{2+}-binding site: six oxygen-containing ligands at positions 1, 3, 5, 7, 9, 12, an invariant glycine at position 6, and a conserved aliphatic residue at position 8.

The presence of four Ca^{2+}-binding sites in CLD explains the observation that Ca^{2+} directly binds to CDPK and regulates its activity. The Ca^{2+}-binding activity of CDPK has been observed with CDPKs purified from soybean, groundnut, winged bean, and *Paramecium* in the electroblot overlay assay with $^{45}Ca^{2+}$ (DasGupta, 1994; Harmon et al., 1987; Saha and Singh, 1995; Son et al., 1993). Mutants of *Arabidopsis* CDPK with different numbers of EF hands (0, 1, 2, 3) were assayed for kinase activity as well as calcium dependence (Hong et al., 1996). The deletion mutants had only basal level activity and lost their calcium dependent activation, which indicates that EF hands are responsible for the calcium-dependent activity.

Several DNA sequences with similar primary structure of CDPK but with different carboxyl terminal domain have been cloned recently. One group of these kinases is CDPK-related kinases (CRK). CRKs from carrot and maize (Furumoto et al., 1996; Lindzen and Choi, 1995) are similar to CDPK but with different carboxyl terminal domains. Furthermore, residues essential for calcium binding in the four EF-hands have not been conserved. A recombinant maize CRK is active *in vitro* without calcium as an activator (Furumoto et al., 1996). The function of carboxyl terminal domain in CRK remains to be elucidated. Another plant kinase related to CDPK is calcium-binding calcium/calmodulin-dependent protein kinase (CCaMK) from lily. CCaMK has a catalytic domain, an autoinhibitory, calmodulin-binding domain, and a neural visinin-like domain (Patil et al., 1995; Ramachandiran et al., 1997; Takezawa et al., 1996).

Recombinant CCaMK directly binds Ca^{2+}. Furthermore, CCaMK binds CaM in a Ca^{2+}-dependent manner (Patil et al., 1995). The visinin-like domain contains three EF-hand motifs which can bind Ca^{2+}, and deletion of this domain abolished the Ca^{2+}-stimulated autophosphorylation activity, which increases Ca^{2+}/calmodulin-dependent protein kinase activity (Takezawa et al., 1996). Since plants have these unique CDPKs and CDPK-related protein kinases, it is important to understand the mechanisms of these kinases and how plants use different regulatory mechanisms to sense calcium changes trigged by external signals.

The calcium-binding properties of CDPK from *Plasmodium* (PfCPK) have been studied by equilibrium dialysis (Zhao et al., 1994). The data demonstrate that one molecule of PfCPK binds four calcium ions with a mean K_d of about 80 μM. The kinase activity is strongly calcium dependent and the calcium concentration required for half-maximal kinase activity ($K_{0.5}$) is 15 μM. Recently the method of flow dialysis was used to determine the calcium-binding properties of three CDPK isoenzymes from soybean (Lee et al., 1998). The K_d's for calcium in the absence of substrates are 51, 1.4, and 1.6 μM for CDPK$_{\alpha, \beta,}$ and $_{\gamma}$ respectively; whereas in the presence of a peptide substrate the K_d of CDPK$_{\alpha}$ decreases to 0.6 μM. The $K_{0.5}$' s of CDPK$_{\alpha, \beta,}$ and $_{\gamma}$ for calcium with peptide substrate syntide-2 are 0.06, 0.4, and 1 μM. The different calcium-binding properties of CDPK provide evidence that each CDPK plays a distinct role in calcium signal transduction.

Biochemical Properties of CDPK

CDPK has a unique structure with the calmodulin-like domain fused with the kinase domain through the junction domain. CDPK has its own calmodulin-like regulatory domain, its calcium-dependent activity is not affected by exogenous calmodulin (CaM), while calmodulin antagonists, such as phenothiazines, W-7 and calmidazolium, inhibit CDPK activity. In general, CDPK depends on calcium and does not require CaM or phospholipids for activity (DasGupta, 1994; Gundersen and Nelson, 1987; Harmon et al., 1987; MacIntosh et al., 1996; Putnam-Evans et al., 1990; Roberts and Harmon, 1992; Saha and Singh, 1995; Son et al., 1993). However, several CDPKs, such as oat plasma membrane CDPK, showed the fatty acids-stimulated activity (Roberts and Harmon, 1992). An *Arabidopsis* recombinant CDPK AK-1 was stimulated synergistically by calcium and phospholipids (Binder et al., 1994; Harper et al., 1993). The nature of the lipid stimulation is waiting to be solved.

Endogenous Substrates of CDPK

While data on the sequences of CDPK are increasing, the physiological role of CDPK is uncertain. Identification of CDPK endogenous substrates should provide valuable information about the function(s) of CDPK. The phosphorylation of plasma membrane H^+-ATPase from oat roots by a membrane-associated CDPK may stimulate the activation of H^+-ATPase (Schaller and Sussman, 1988). Tonoplast intrinsic protein (TIP), a putative water channel, is phosphorylated by an *Arabidopsis* CDPK (Johnson and Chrispeels, 1992). The cytosolic Ca^{2+} concentration increases in guard cells when plants are stressed and

the increased Ca^{2+} concentration regulates the activities of ion transporters. This implicates that calcium-mediated signal transduction and calcium-dependent protein phosphorylation contribute to the regulation of stomatal aperture of guard cells (Assmann, 1993). Pei et al. (1996) showed a constitutively active *Arabidopsis* CDPK activated a tonoplast chloride channel in isolated vacuoles from *Vicia faba*, but it is unclear whether CDPK directly phosphorylates the ion channel or phosphorylates an intermediary protein(s). Recently, Li et al. (1998) reported that guard cells possess a CDPK that phosphorylates the KAT1 potassium channel. It is convincing that CDPK is involved in the Ca^{2+}-regulated modulation of plasma membrane ion channels in guard cells. Sucrose-phosphate synthase and NADH: nitrate reductase are found to be regulated by reversible phosphorylation through calcium-dependent protein kinases (Bachmann et al., 1996; Huber et al., 1996; Huber et al., 1994; McMichael et al., 1995). Nodulin-26, an integral symbiosome membrane transporter of nitrogen-fixing soybean nodules is phosphorylated by a CDPK of soybean root nodules (Weaver and Roberts, 1992; Weaver et al., 1994). Site-directed mutagenesis of serine-262 indicates that phosphorylation of this residue modulates channel activity by conferring voltage sensitivity (Lee et al., 1995).

The substrate specificity has been described for CDPKs from different plants (Roberts and Harmon, 1992). Most substrates used for CDPK assays are arbitrary substrates such as histone H1, casein, and synthetic peptides derived from glycogen synthase, myosin light chain, ribosomal protein S6, and nodulin 26. CDPKs phosphorylate proteins or peptides with a basic-X-X-Ser/Thr motif (X stands for any amino acid, the basic amino acid can be either Arg or Lys). Certain amino acid residues other than the core motif sequence were found to

determine the substrate preference for some CDPK isozymes. Bachmann et al. (1996) reported that partially purified CDPK from spinach was able to phosphorylate nitrate reductase at Ser-543. Synthetic peptides with various residues surrounding the phosphorylation site were analyzed and showed that peptide substrates with a Leu-X-basic-X-X-Ser motif are preferred by the spinach CDPK. One CDPK isoform from soybean, $CDPK_{\alpha}$, was shown to prefer peptide substrates with aliphatic amino acid residues at positions P-5, P+1, and P+4 (P stands for phosphorylation site, - stands for amino side, + stands for carboxyl side) (Lee et al., 1998). Further study is needed to define the residues outside of the core motif that are important for the substrate specificity.

Ca^{2+} Regulation in Plant Cells and Its Role in Signal Transduction

Calcium Regulation

Environmental and hormonal stimuli affect many aspects of growth and developmental processes in plants. Many external stimuli cause changes, usually a rise, in cytosolic Ca^{2+}. Ca^{2+}, as an intracellular messenger, acting through Ca^{2+}-modulated proteins and their targets, regulate numerous cellular processes. Studies of Ca^{2+}-based signal transduction have been advanced by the development of methods to measure the concentration of the cytosolic Ca^{2+} in plants. Ca^{2+}-selective microelectrodes have been used extensively to measure changes in cytosolic $[Ca^{2+}]$. However, the development of fluorescent indicator dyes (Grynkiewicz et al., 1985; Haugland, 1992; Tsien and Poenie, 1986) and photoprotein aequorin (Knight et al., 1991a; Knight et al., 1991b; Knight et al.,

1992; Shimada et al., 1991) allow quantification of spatial and temporal kinetics of cytosolic [Ca^{2+}]. The measured cytoplasmic [Ca^{2+}] in unstimulated, resting cells is about 30-200 nM, although the extracellular [Ca^{2+}] and [Ca^{2+}] inside the vacuole and endoplasmic reticulum (ER) are in the range of millimolar.

The ability of the cells to maintain low cytosolic [Ca^{2+}] and to response to external signals with changes in cytosolic [Ca^{2+}] is due to the combined activities of Ca^{2+} transport proteins. There are two classes of transporters: efflux transporters, mediating Ca^{2+} efflux from the cytoplasm, including the Ca^{2+}-ATPase and Ca^{2+}/nH^{+} antiporters; Ca^{2+} channels, mediating Ca^{2+} influx to the cytoplasm. Ca^{2+}-ATPases have been found in the plasma membrane (PM), tonoplast (TN) and ER of plant cells. The functions of Ca^{2+}-ATPases seem to maintain the steady-state Ca^{2+} (Evans et al., 1991) and restore cytosolic Ca^{2+} to prestimulated level (Felle et al., 1992; Hepler and Wayne, 1985). Ca^{2+}/nH^{+} antiporter activity is mainly associated with TN. *In vitro* characterization and direct measurement of cytosolic Ca^{2+} support the role of antiporter in damping large changes in cytosolic Ca^{2+} and maintaining the Ca^{2+} store in the vacuole (Bush, 1995). Ca^{2+} channels are found on PM and TN. There are three classes of Ca^{2+} channels in plants: voltage-operated channels located on the PM and TN; inositol-1, 4, 5-triphosphate (IP_3)-operated channels located on the TN; and stretch-operated channels on the PM. Unlike in animals, IP_3-operated channels have not been identified on the ER and there is no evidence for the IP_3-induced release of Ca^{2+} from the ER in plants (Bush, 1995). Current data indicate that the primary routes of stimulus-induced Ca^{2+} influx into the cytosol are through the Ca^{2+} channels on the PM and TN.

The combined activities of Ca^{2+} efflux and influx transporters provide overall regulation of the efflux and influx of Ca^{2+} leading to changes in cytosolic Ca^{2+}.

Ca^{2+} and Signaling Pathways

Calcium plays an important role in stimulus-response coupling in all eukaryotic organisms. In plants, Ca^{2+} regulates a range of cellular functions from ionic balance, motility, and carbohydrate metabolism to gene expression, secretion, and mitosis (Bush, 1995). Although Ca^{2+} is not the only regulator in these processes, any stimulus that induces a complex cellular response will likely involve Ca^{2+} at some point in the signal transduction pathway.

To establish the role of Ca^{2+} in the signal transduction pathway, information about the change of Ca^{2+} upon stimulation and the target proteins that are activated by the changed Ca^{2+} is needed. Cytosolic Ca^{2+} changes have been reported following many different stimuli. Plant hormones such as auxin, ABA, and GA induce transient increases, sustained increases or oscillations in cytosolic Ca^{2+} (Bush and Jones, 1988; Felle, 1988; Gehring et al., 1990a; Gilroy et al., 1991; Gilroy and Jones, 1992; Irving et al., 1992; McAinsh et al., 1990; McAinsh et al., 1992; Schroeder and Hagiwara, 1990). Elicitors and numerous physical stimuli such as cold shock, wind, touch, salinity, gravity, and light induce transient or sustained increases of cytosolic Ca^{2+} (Gehring et al., 1990b; Knight et al., 1997; Knight et al., 1991b; Knight et al., 1992; Lynch et al., 1989; Shacklock et al., 1992). The discovery of the target proteins of the elevated Ca^{2+} is a key element to understanding Ca^{2+}-mediated signal transduction. In plants, biochemical and molecular evidence indicates that CDPK is the primary calcium-

regulated protein kinase, although there is evidence for the presence of Ca^{2+}/CaM-dependent kinase, and Ca^{2+}/phospholipid-dependent kinase. Ca^{2+}-dependent phosphatase also has been identified in plants (Bush, 1995). A Ca^{2+}/CaM-dependent nitric oxide synthase was implicated for the synthesis of the nitric oxide signal in disease resistance in plants (Delledonne et al., 1998). To date, much progress has been made in identifying the function of Ca^{2+} in the transduction of a variety of stimuli. The red light response, ABA and guard cell closure, and GA and secretion will be reviewed below to demonstrate the involvement of Ca^{2+} in the signal transduction pathways.

Red light response—phytochrome-mediated signal transduction

The response of plants to red light is mediated by phytochrome. Phytochrome is a well-characterized plant photoreceptor (Quail et al., 1995), able to modulate many morphological, physiological, and biochemical events through a still undefined mechanism. Although a role for Ca^{2+} in phytochrome signaling was proposed in 1980s (Roux, 1984; Roux et al., 1986), the direct observation of a red light-induced Ca^{2+} transient increase was reported in plant cells (Shacklock et al., 1992) much later. In etiolated wheat leaf protoplasts, red light induces swell. Swelling is preceded by a transient increase in free calcium in cytosol, which later decreases to below the resting level. The photolytic release of caged calcium and inositol 1, 4, 5-triphosphate induced a transient increase in Ca^{2+} and a subsequent increase in protoplast volume.

Recently, using biochemical and cell biological approaches, Chua and colleagues microinjected various signaling molecules, agonists, and antagonists into the cells of a phytochrome-deficient tomato mutant (Bowler et al., 1994;

Neuhaus et al., 1993). The injection of PhyA into single cells of the tomato mutant aurea restored wild-type characteristics. The microinjection of GTPγS, a nonhydrolyzable GTP analog that activates G proteins, identified one or more heterotrimeric G proteins as the very early element in the phytochrome phototransduction. Whereas, microinjected Ca^{2+} and activated calmodulin (Ca^{2+}/CaM) can stimulate the expression of a photoregulated cab-GUS reporter gene together with the synthesis and assembly of some, but not all, of the photosynthetic complexes. The other phytochrome-mediated pathway, such as biosynthesis of anthocyanin, is not calcium dependent. However, cyclic GMP was found to mediate PhyA-dependent anthocyanin biosynthesis and to participate with Ca^{2+} and Ca^{2+}/CaM in coordinating chloroplast development. The data strongly suggest that through an unknown mechanism phytochrome may activate G protein, which stimulates increases in the cytosolic level of cGMP and Ca^{2+}, which in turn activates parallel and partially convergent signaling pathways that induce the changes in gene expression.

Protein phosphorylation cascades have long been postulated as a red light signaling transduction mechanism. Fallon et al. detected very rapid red light-induced calcium-sensitive protein phosphorylation in etiolated wheat leaf protoplasts (Fallon et al., 1993). There are also other reports of the light-modulated phosphorylation of various proteins (Fallon and Trewavas, 1994; Harter et al., 1994), but conclusive evidence is still lacking. The establishment that higher plant oat and green alga phytochromes are Ser/Thr protein kinases (Yeh and Lagarias, 1998) provides one more piece of evidence for phytochrome-mediated protein phosphorylation in the transduction of light signals in plants. Recombinant phytochromes from oat and a green alga exhibit Ser/Thr kinase

activity similar to that of phytochrome isolated from dark-grown seedlings. It is very exciting to predict that the finding of the substrate or interaction protein of phytochrome will open the "black box" of the red light signal transduction pathway.

ABA and guard cell closure

Guard cells are a favorite system for studying how external signals are transduced to the internal cells and how they alter cell behaviors. This is partly because guard cells respond to an array of stimuli, including ABA, auxin, red/blue light, and humidity. In addition, responses to the stimuli are easily detected as a change in guard cell volume and therefore stomata aperture; plus guard cells in epidermal peels of many plants (especially *Vicia faba* and *Commelina communis*) are readily available.

ABA has been known to induce stomatal closure during water stress. The application of ABA to guard cells results in a rapid rise in cytosolic Ca^{2+} that precedes stomatal closure (Gilroy et al., 1991; Irving et al., 1992; McAinsh et al., 1990; McAinsh et al., 1992). The release of Ca^{2+} from a caged form in the cytosol is sufficient for stomatal closure (Gilroy et al., 1990). However, under certain conditions, ABA can induce stomatal closure via a Ca^{2+}-independent mechanism (Allan et al., 1994).

Stomatal closure requires depolarization of the plasma membrane and efflux of K^+ and anions (Assmann, 1993). Increases in cytosolic Ca^{2+} regulate several ion transporters that are essential in the control of stomatal aperture (MacRobbie, 1997; McAinsh et al., 1997). The plasma membrane proton pump of *V. faba* guard cells, which hyperpolarizes the plasma membrane and thus

provides the driving force for K^+ and Cl^- uptake, is inhibited by increased cytosolic calcium (Kinoshita et al., 1995). The inward K^+ channels in the PM of *V. faba* guard cells, which are responsible for K^+ influx, are also inhibited by increased cytosolic Ca^{2+} (Schroeder and Hagiwara, 1989). Otherwise, a type of K^+ channel in the tonoplast of *V. faba* guard cells, which may control K^+ efflux from guard cell vacuoles during stomatal closure, is activated by elevated cytosolic Ca^{2+} (Ward and Schroeder, 1994). Thus elevated Ca^{2+} appears to coordinate K^+ efflux by inactivation of the inward K^+ channel at the PM and by activation of an outward K^+ channel at the TN. In addition, both slow-type and rapid-type plasma membrane anion channels, which allow Cl^- and malate efflux during stomatal closure, are also activated by increased cytosolic Ca^{2+} (Hedrich et al., 1990; Schroeder and Hagiwara, 1989).

Despite the effects of increased cytosolic Ca^{2+} in guard cells on ion transporters, the signaling steps from elevated Ca^{2+} to the ion fluxes are not completely understood, but the data indicate that phosphorylation/dephosphorylation plays a role in the signal transduction. The effects of Ca^{2+} on the inward K^+ channel at the PM may be mediated through a homolog of a Ca^{2+}-dependent protein phosphatase calcineurin (Luan et al., 1993) and may involve the action of heterotrimeric GTP-binding proteins (Fairley-Grenot and Assmann, 1991). Recently, Li et al. reported that *V. faba* guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 K^+ channel in a Ca^{2+}-dependent manner (Li et al., 1998). However, the effect of phosphorylation of KAT1 on its activity has not been established. KAT1 is a plant K^+ channel gene initially cloned from *Arabidopsis* (Anderson et al., 1992). Electrophysiological and transgenic studies indicated that the KAT1 protein is

very likely to be the guard cell inward K^+ channel whose activity is inhibited by elevated cytosolic Ca^{2+} concentration (see references in Li et al., 1998). Further evidence is needed in order to clarify the roles of G-protein, calcium-dependent phosphatase, and protein kinase in regulating inward K^+ channel upon elevated cytosolic Ca^{2+}. Additionally, an *Arabidopsis* CDPK activated a tonoplast Cl^- channel in isolated vacuoles from *V. faba* guard cells (Pei et al., 1996). Since CDPKs belong to a large gene family, it is possible that different CDPK isoforms regulate different substrates, such as K^+ or Cl^- channel, and signal transduction pathways. Although other factors are important for regulating guard cell volume, the role of Ca^{2+} as a messenger that coordinates ion transports appears essential.

GA and secretion in cereal aleurone cells

The cereal aleurone cell is well established as a model system for studying the regulation of plant cells by GA (Jones and Jacobsen, 1991). Aleurone cells secret hydrolyases (predominantly α-amylases) that mobilize endosperm reserves during seed germination. GA stimulates α-amylases synthesis and secretion. Considerable progress has been made in understanding the role of Ca^{2+} in the transduction of GA signal in aleurone cells. More data support that both calcium-dependent and calcium-independent pathways exist in GA signal transduction. A rise in cytosolic Ca^{2+} has been observed in wheat aleurone cells and barley aleurone protoplasts after GA treatment (Bush, 1996; Gilroy and Jones, 1992). The activation of a calcium influx channel on the PM and the reduction in activity of PM Ca^{2+}-ATPase contribute to the elevated cytosolic calcium. Induction of mRNA of α-amylase by GA does not require elevated

cytosolic Ca^{2+}, which indicates that Ca^{2+} may not be involved in the GA action leading to the expression of α-amylase gene (Gilroy, 1996).

The secretion of α-amylase in aleurone cells upon GA treatment is mediated through an increase in cytosolic Ca^{2+} (Gilroy, 1996). In the wheat aleurone, GA induces a steady-state increase in Ca^{2+} of 500 nM. This increase is initiated immediately after GA addition and is completed within 30-90 minutes (Bush, 1996). A similar but prolonged increase is observed in barley aleurone protoplasts (Gilroy, 1996; Gilroy and Jones, 1992). However, mimicking GA-induced changes in Ca^{2+} did not mimic GA action, while blocking the changes in Ca^{2+} prevented the GA-stimulated secretion of α-amylase (Gilroy, 1996). The identification of target proteins of GA-induced increased cytosolic Ca^{2+} is the key to understanding the mechanism of Ca^{2+} regulation. CaM and Ca^{2+}/CaM-regulated Ca^{2+}-ATPase in the ER, Ca^{2+}/CaM slow-vacuolar channel, and CDPK are the potential targets of elevated cytosolic Ca^{2+}. Following GA stimulation, a Ca^{2+}/CaM-regulated Ca^{2+}-ATPase in the ER becomes activated (Bush et al., 1989; Gilroy and Jones, 1993), this provides Ca^{2+} for the formation of α-amylase, in which Ca^{2+} is a structural component. The Ca^{2+}/CaM slow-vacuolar channel is found on the storage protein vacuole of the aleurone cell and is activated by increasing cytosolic Ca^{2+} above 600 nM, the channel activity is further stimulated by exogenous CaM (Bethke and Jones, 1994). Recently, a calcium-dependent, calmodulin-independent protein kinase has been identified by cross-linking of microinjected photoaffinity-labeled peptide substrate—syntide-2 into barley aleurone protoplasts (Ritchie and Gilroy, 1998). Syntide-2 selectively inhibited the GA response, leaving constitutive and abscisic acid-regulated events

unaffected. This implicates that CDPK acts as a calcium-dependent regulator of the GA response in aleurone cells.

There is overwhelming evidence for the role of calcium in regulating cellular functions in response to many stimuli in plant cells. As a predominant calcium-regulated protein kinase in plants, CDPK has been implicated in many signal transduction pathways. Defining these signal transduction pathways is still challenging and will be a major step toward understanding the role of calcium in signaling in plant cells.

CHAPTER 2
IDENTIFICATION OF PEPTIDE SUBSTRATES OF CDPK FROM RANDOM PEPTIDE PHAGE DISPLAY LIBRARIES

Introduction

Soybean CDPK (Calmodulin-like domain protein kinase) is a member of a large family of protein kinases that contain a catalytic domain and a calmodulin-like domain that are separated by the junction domain (Roberts and Harmon, 1992). DNA encoding CDPKs have been cloned from soybean and other plants such as *Arabidopsis*, carrot, rice, corn, mungbean, and also from algae and protists. While many CDPK genes have been identified, elucidation of the physiological roles of CDPKs has been slow. Only a few endogenous substrates have been identified. Although CDPKs phosphorylate a variety of synthetic peptides having a basic-X-X-Ser/Thr core motif (Roberts and Harmon, 1992), residues outside the motif that are important for the substrate specificity have not been characterized. Physiological studies would be aided if a specific substrate or inhibitor of CDPK were available. The identification of a CDPK substrate or inhibitor can be achieved by screening a random peptide phage display library.

Phage display has rapidly developed as a technology for the identification of novel peptide ligands or protein domains that bind to diverse targets (Barbas, 1993; Bradbury and Cattaneo, 1995; Clackson and Wells, 1994; Smith and

Petrenko, 1997; Smith and Scott, 1993; Wells and Lowman, 1992). Since the 1980s, Smith (1985) first demonstrated that an *E. coli* filamentous phage M13 could express a fusion protein bearing a foreign peptide on its surface. These foreign polypeptides were accessible to antibodies so that the fusion phage with a foreign peptide binding a specific antibody could be enriched over ordinary phage by a powerful selection technique: affinity purification (Smith, 1985). It was suggested that libraries of random peptide phage display might be constructed and screened for epitopes of antibodies and interacting peptides of other proteins. In early studies, three libraries of random peptide sequences fused to the N-terminus of a minor coat protein pIII of a filamentous phage were constructed and screened by affinity selection. The identified sequences bound specifically to the cognate antibodies or streptavidin (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990).

The applications of phage display were broadened by the construction of libraries of protein, especially antibody, displayed on the phage. These libraries have been used to modify the properties of proteins, study protein-protein interaction, and generate monoclonal antibodies. Phage display and affinity purification are useful tools for identifying and modifying substrates and inhibitors of enzymes and searching for ligands of target molecules for the purpose of drug development.

Ff Phage Biology

M13, fl, and fd are collectively referred to as the Ff phage because of their filamentous appearance and their dependence on the F plasmid for the infection

of host *Escherichia coli*. Ff phage has a single-stranded, covalently closed DNA genome that is encased in a capsid that is approximately 7 nm wide by 900 to 2000 nm in length. The phage capsid is comprised of about 2700 copies of the major coat protein pVIII and closed at two ends by 4 or 5 copies of each of four species of minor coat proteins (pIII, pVI, pVII, pIX). Ff phage genomes have been completely sequenced and are more than 98% identical (Beck et al., 1978; Beck and Zink, 1981; van Wezenbeek et al., 1980). Ff phage infects *Escherichia coli* containing the F conjugative plasmid. Unlike lytic phages, which are released by cell lysis after they have been assembled in the host cell cytoplasm, Ff phage are extruded continuously through host cell cytoplasmic membrane where the inner and outer membranes are in close contact and where assembly occurs.

Infection begins when the tip of the phage particle attaches to the tip of the host cell F pilus. After the major capsid proteins integrate into the host membrane and the phage DNA (+strand) is translocated into the cytoplasm, bacterial polymerases synthesize the complementary strand (–strand) and convert the infecting phage ssDNA into a supercoiled, double-stranded circular replication form DNA (RF). This molecule serves as a template for transcription and translation from which all of the phage proteins are synthesized. Some of the phage proteins, in concert with host enzymes, direct the synthesis of single-stranded phage DNA that is converted to additional RF. When the phage specific single-stranded DNA-binding protein pV reaches the proper concentration, it sequesters the newly synthesized single-stranded phage DNA. The DNA in this pV-binding form is not converted to RF but rather is assembled into new phage particles (virions).

Assembly and export of phage is a membrane-associated process which takes place at specific assembly sites where the inner and outer membrane are in close contact (Lopez and Webster, 1985). The capsid major protein (pVIII) (Wickner, 1975), minor proteins (pIII, pVI, pVII, and pIX) (Endemann and Model, 1995), and other phage proteins involved in the assembly of the particle become integrated into the cytoplasmic membrane. During the assembly process, single-stranded DNA-binding proteins (pV) are displaced and capsid proteins assemble along the DNA as it is extruded through the membrane envelope. The transfer of the capsid proteins from their cytoplasmic membrane location to the assembling phage particle is directed by three phage noncapsid assembly proteins and the bacterial protein thioredoxin (Russel, 1991). Coat protein pVI and pIII are added to the end of the extruding virion, and the phage is released into the media.

pIII is required not only for F pilus adsorption but also for terminating virion assembly and stabilizing the viral particle. pIII is synthesized with an 18-residue amino-terminal signal sequence and requires the bacterial Sec system for insertion into the cytoplasmic membrane (Rapoza and Webster, 1993). After the removal of the signal peptide, the mature 406 amino acid residue protein spans the membrane once with five carboxyl-terminal residues in the cytoplasm (Davis et al., 1985), the amino-terminal 379 residues are placed in the periplasm. The amino-terminal region mediates phage adsorption and DNA penetration, while the carboxyl-terminal part functions in virion assembly and structure.

Infection of *E. coli* by the Ff bacteriophage is a two-step process (Model and Russel, 1988). The first step involves the interaction of the pIII end of the phage particle with the tip of the F conjugative pilus. Retraction of the pilus,

presumably by depolymerization of pilin subunits into the inner membrane, brings the tip of phage to the membrane surface (Frost, 1993). The second step involves the integration of pVIII major coat proteins and perhaps the other coat proteins into the inner membrane, together with the translocation of DNA into the cytoplasm. The translocation event requires the products of bacterial tolQRA genes (Russel et al., 1988; Webster, 1991). Recognition of the receptor at the pilus tip and the appropriate Tol proteins appears to be a function of the amino-terminal region of pIII. The removal of the amino-terminal 36kD of pIII renders the phage particle noninfectious (Armstrong et al., 1981; Gray et al., 1981). The analysis of a set of deletion mutants in gene III suggests that separate regions of the pIII protein are involved in the pilus adsorption and TolQRA-dependent penetration steps (Stengele et al., 1990). The pilus recognition site is present in residue 99-196, while the residue 53-107 region is involved in the penetration of DNA into the bacterium. It is not known whether the adsorption and penetration domains must be contiguous for pIII to function in the infection. It is best to fuse peptides or proteins at or near the amino-terminus of pIII to create an infectious phage particle.

Phage Display and Application

Vectors of surface display

In the construction of phage display library, oligonucleotides or foreign genes are usually inserted into the 5′ end of gIII and gVIII because protein pIII and pVIII have surface-exposed amino-terminal domains that tolerate foreign

peptide inserts. Phage display can be classified as Type 3, 33, 3+3, 8, 88, 8+8 according to the use of the coat protein pIII or pVIII and the number of products.

A type 3 vector has a single recombinant gene III (gIII) bearing a foreign DNA, each of the five copies of pIII display the foreign peptide encoded by the insert DNA. Some large foreign peptides are not well tolerated in Type 3 vectors because they interfere with pIII function by promoting periplasmic degradation or hindering phage infection or assembly. Type 33 vector alleviates this problem by having two gIIIs, one wild type, and the other recombinant with foreign DNA. The virions display a mixture of pIII molecules, only some of pIII are fused to the foreign peptide and incorporated to some degree into the virion, the remaining wild type pIII are sufficient for assembly and infection. Type 3+3 vector also has two gIIIs, but the recombinant gIII is on a phagemid. The phagemid contains the Ff phage intergenic region that includes origins of replication and packaging signal in addition to a plasmid replication origin and an antibiotic resistance gene. When phagemid-harboring cells are superinfected with a helper phage, the phage replication and assembly machinery, together with the cis-acting signals in the intergenic region, package the phagemid genome as well as the helper genome into virions. Therefore, two types of phage particles—helper and phagemid are secreted. Both of them incorporate a mixture of wild type and recombinant pIII molecules.

Type 8, 88, and 8+8 vectors are the gVIII counterparts of gIII vector Type3, 33, and 3+3. Type 8 vectors can display only five or six amino acid residues, longer peptides have been displayed as Type88 (Huse et al., 1992) and 8+8 (Barbas et al., 1991; Kang et al., 1991).

Applications and innovations in phage display library

Phage display has rapidly matured as a widespread technology for its linking the phenotype of a bacteriophage surface-displayed peptide with the genotype encoding that peptide, packaged within the same phage particle. Foreign sequences can be inserted into gene III or gene VIII to generate a library of fusion phage. Large numbers of virions only occupy a small volume ($>10^{12}$ particles/mL) makes screening of a diverse library achievable. The library can be screened to identify specific phage that display any sequence for which there is a binding partner, such as an antibody, antigen, or enzyme. The screening is performed by a series of affinity purification, known as biopanning. In each panning, phages are incubated the target, which is immobilized on a solid matrix. Phages that do not bind are washed away, and bound phages are eluted and amplified for the next panning. After several rounds of panning, phages with tight-binding peptide will be enriched. Since its inception in 1985 (Smith, 1985), phage display library has been generated to display random peptide, gene fragment, protein, especially antibody and used in research areas such as epitope mapping, enzyme inhibitor/substrate study, protein-protein interaction, and antibody production.

Phage-displayed protein and antibody library. Longer polypeptides have been displayed in type 33, 3+3, 88, and 8+8 vectors. A few proteins were displayed in type 3 vector (Soumillion et al., 1994) with low infectivity and considerable proteolysis (Gram et al., 1993; McCafferty et al., 1991; Roberts et al., 1992b; Swimmer et al., 1992). An early protein display paper described phagemid display of the 191-residue disulfide-containing hGH, accomplished by

fusion of hGH to the pIII C-terminal domain ($pIII_C$) in a type 3+3 vector (Bass et al., 1990). Importantly, hGH-$pIII_C$ could bind to hGH receptor and was recognized by monoclonal antibodies whose epitopes are sensitive to hGH conformation. Many proteins have now been displayed and contain their biological functions (Wilson and Finlay, 1998).

Phage display is popular for the *in vitro* selection of peptides and proteins with altered properties. A study on bovine pancreatic trypsin inhibitor (BPTI), termed "directed evolution of a protein", illustrates the selection approach (Roberts et al., 1992a). A phage display library with the size of 1,000 member was constructed by limited randomization of five residue positions of BPTI. The library was biopanned with immobilized human neutrophil elastase (HNE) to yield a variant with more than 106-fold affinity for HNE than the parental BPTI sequence. Other studies have yield variant clones with altered target specificity. These include not only other protease inhibitors (Dennis and Lazarus, 1994a; Dennis and Lazarus, 1994b; Wang et al., 1995) but also an enzyme with the altered specificity for active site ligands (Widersten and Mannervik, 1995) and zinc fingers with modified specificity (Choo and Klug, 1995).

The applications of phage display were broadened by the construction of libraries of single-chain antibody displayed on phage. The principle of antibody display was first demonstrated by McCafferty et al. (McCafferty et al., 1990). The heavy- and light-chain variable domains of anti-lysozyme antibody were linked on the same polypeptide and expressed as gene III fusion protein. Over 1,000-fold enrichment of antibody could be obtained by a single passage over a lysozyme-Sepharose column. This method was then developed to allow the display of libraries of combining domains such that new antibodies or mutant

versions of existing antibodies could be generated (Griffiths and Duncan, 1998; Hoogenboom, 1997; Hoogenboom et al., 1998; Rader and Barbas, 1997). Antibody phage display has revolutionized the generation of monoclonal antibodies, particularly human antibodies (Aujame et al., 1997). It is now possible to create antibodies to antigen with high affinity (nanomolar to picomolar range) without the use of laboratory animals or hybridomas.

Random peptide library and application. Random peptide libraries (RPLs) with the length of 6-38 amino acid displayed have been described. The number of randomized residues determines the complexity of the library. The number of possible sequences within a peptide region is a function of the peptide length, and can be roughly predicted by $c = 20^n$, where c represents the number of unique amino acid sequences, 20 reflects the number of different amino acids possible in each position, and n refers to the number of residues contained in the random sequence. For example, a hexapeptide library contains $20^6 = 6.4 \times 10^7$ unique sequences.

The large number of peptides in a library constitutes an essential feature that determines its potential usefulness. The minimum number of unique clones in a phage display library containing all peptides of a particular length can be calculated as x^n, n refers to the number of residues in the random sequence, and x is the number of possible codons in the encoding oligonucleotide. Usually 32 codons (x = 32) are used in the form of NNS or NNK (N stands for G, C, A, or T; S stands for G or C; and K stands for G or T) to reduce the bias inherent in the genetic code. Thus a library displaying all possible hexapeptides must contain at least 32^6 (1.1×10^9) different phage particles. Assuming a practical upper limit of 10^{10} clones in a library, libraries with more than six residues risk being

incomplete. But libraries with relatively long peptides may have advantages because several shorter random peptides in different flanking sequence contexts are contained within each clone, thus, they may be more useful if the interest is to defining binding preferences rather than extract a consensus sequence.

Random peptide phage display library has been used for epitope mapping through affinity selection with antibodies. A successful example is the identification of the native epitope of an anti-human immunodeficiency virus-1 monoclonal antibody (Conley et al., 1994). In this study, affinity selection from a 15-residue RPL with the antibody yielded 20 clones which could be classified into four groups with displayed consensus sequences $ELD^{R}/_{K}W$, $EXD^{R}/_{K}W$, $XLD^{R}/_{K}W$, XXDKW, and thus ELDKW was identified as the native epitope. Not all RPL studies yield such apparent success, but usually RPL selection will yield at least a compositional theme rather than a position-specific consensus sequence (Bottger and Lane, 1994; Bottger et al., 1995; Sioud et al., 1994).

Affinity selections from RPLs with non-antibody molecules have been used to define protein binding domain motif, and substrate preferences. Affinity selection from 6- and 15-residue RPLs with the integrin $\alpha_v\beta_3$ enriched a collection of sequences containing the RGD triplet (Healy et al., 1995). Similarly, two groups independently biopanned RPLs with fusion proteins containing Src SH3 domain, identified similar proline-containing sequences related to a known SH3 domain binding motif (Cheadle et al., 1994; Sparks et al., 1994). In contrast, biopanning from a 6-residue RPL with S-protein (a fragment of bovine pancreatic ribonuclease) yielded a sequence motif of $^{F}/_{Y}NF^{E}/_{V}{}^{I}/_{V}{}^{L}/_{V}$ that bore little resemblance to S-protein's natural ligand. Affinity panning of RPLs with the molecular chaperone BiP revealed a loose fitting binding motif of BiP. The motif,

$\Phi^{W}/_{X}\Phi X\Phi X\Phi$ (Φ = a large hydrophobic residue, X = any amino acid residue), fits well with the role of BiP in recognizing partially folded polypeptides (Blond-Elguindi et al., 1993). Two different peptide motifs were selected in another affinity selection of RPL with Hsc70 (70-kilodalton heat shock cognate protein, a constitutively expressed member of a family of peptide/unfolded protein-stimulated ATPases—the Hsp70 family of molecular chaperones). It is proposed that Hsc70 recognizes two different amino acid sequence motifs in its dual roles of chaperoning proteins to organelles and facilitating protein folding (Takenaka et al., 1995).

Affinity selections of RPLs with calmodulin (CaM) have identified CaM-binding motifs in which the overall sequences are different, but they all bear Tryptophan (W) at the N-terminus of the peptides (Adey and Kay, 1996; Dedman et al., 1993; Nevalainen et al., 1997). The catalytic specificity of four protein tryosine kinases (PTKs) Blk, Lyn, c-Src, and Syk was assessed by phage display library (Schmitz et al., 1996). A diverse peptide display library was established, and after multiple rounds of phosphorylation and selection of phage displaying phosphotryosine-containing peptides, canonical substrate sequences for each PTK were enriched. The highly related Src-like PTKs c-Src, Blk, and Lyn share a common substrate preference, they also have their own substrate preferences. Sky, not belonging to the Src-like PTK family, revealed a distinct substrate requirement.

In an effort to define the substrate preferences of CDPK and identify a specific inhibitor of CDPK, a 15-residue random peptide phage display library was biopanned with $CDPK_{\alpha}$ in this study. One substrate peptide with high affinity was obtained from the affinity selection. A 10-residue random peptide

library based on the peptide substrate sequence obtained in the first screening was constructed and biopanned with $CDPK_{\alpha}$.

Materials and Methods

Cloning and Purification of Recombinant CDPKs

Cloning and purification of biotinylated $CDPK_{\alpha}$

Plasmid construction. PinPoint™ vector Xa-3 (Promega) was used to express biotinylated fusion protein. Plasmid Xa-3 DNA was digested with *Hind* III followed by filling-in with klenow fragment of DNA polymerase I and dephosphorylation by calf intestinal alkaline phosphatase. The plasmid pET1530 containing full-length $CDPK_{\alpha}$ was digested with *Nde* I and *Cla* I followed by filling-in with klenow fragment of DNA polymerase I. Two fragments with the expected sizes from pET1530 and Xa-3 were gel-purified with QIAEX II gel extraction kit (QIAGEN Inc.) according to the manufacturer's protocol, then ligated with T4 DNA ligase and transformed into *E. coli* JM109 cells. Positive clones were verified by restriction enzyme digestion.

Expression in *E. coli*. *E. coli* SG1611 was the host cell for the expression. A single colony was inoculated in LB medium (5 g/L Bacto-yeast extract, 10 g/L Bacto-tryptone, 10 g/L NaCl) with 100 μg/mL ampicillin and 2 μM biotin. The culture was grown until OD_{600} reached 0.4. Cells were collected by centrifugation at 6,500 g for 10 minutes and resuspended in fresh LB medium. The culture was induced by 0.1 mM isopropyl β-D-thiogalacto-pyranoside (IPTG) at 24°C for four

hours. Cells were harvested by centrifugation at 8,000 g for 10 minutes, then stored at -80°C until the purification was performed.

Protein purification. The cells were resuspended in 10 volumes of sonication buffer with protease inhibitors (20 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluride (PMSF), 10 μg/mL leupeptin). The sonication buffer contained 50 mM tris(hydroxymethyl)-aminomethane-HCl (Tris-HCl), pH 7.2, 150 mM NaCl, 2 mM ethylenediaminetetraacetate (EDTA), 0.5 mM dithiothreitol (DTT), 0.1% Triton X-100. All the steps were performed at 4°C. Cell suspension was sonicated according to the manufacturer's instruction. Crude lysate was centrifuged at 10,000 g for 15 minutes to remove cellular debris. The supernatant was applied slowly (flow rate less than 1 mL/min) to a monomeric avidin SoftLink™ column which was equilibrated in sonication buffer. The column was washed with four volumes of wash buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA), salt wash buffer (50 mM Tris-HCl, pH 7.2, 1.0 M NaCl, 2 mM EDTA), and wash buffer consecutively. The protein was eluted with 5 mM biotin. The fractions containing biotinylated $CDPK_{\alpha}$ were pooled and dialyzed against dialysis buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM EDTA) then concentrated by ultrafiltration (Centricon 30, Amicon) and stored in 50% glycerol at -80°C.

Purification of other recombinant CDPKs

GST-$CDPK_{\alpha}$. Full length of $CDPK_{\alpha}$ from plasmid pET1530 was cloned into GST-fusion expression vector PGEX-KG. The expression was performed in bacterial host PR745 cells. A single colony was inoculated in LB medium with 100 μg/mL ampicillin overnight. The overnight culture was diluted 500-fold in

M9TB (10 g/L Bacto-tryptone, 5 g/L NaCl, 1 g/L NH_4Cl, 3 g/L KH_2PO_4, 3.5 g/L Na_2HPO_4, 1 mM $MgSO_4$, 0.4% glucose) medium with ampicinin. The culture was incubated at 37°C until OD_{600} reached 0.4. The expression of the recombinant protein was induced by 0.4 mM IPTG at room temperature. Cells were harvested by centrifugation at 8,000 g for 10 minutes, and the pellet was stored at -80°C.

The expressed GST-CDPK$_\alpha$ was purified through glutathione-agarose column. Briefly, the cell pellet was suspended in sonication buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl). Suspended cells were sonicated and clarified as above. The supernatant was loaded to the glutathione-agarose column, washed with at least five column volumes of high salt wash buffer (50 mM Tris-HCl, pH 7.2, 1.0 M NaCl). GST-CDPK$_\alpha$ was eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. The fractions containing kinase activity were pooled, dialyzed and concentrated.

His$_6$-CDPK$_\alpha$. The expression and purification of His$_6$-CDPK$_\alpha$ was performed according to the procedures published (Harmon et al., 1994; Yoo and Harmon, 1996). *E. coli* hosts for the expression of His$_6$-CDPK$_\alpha$ were BL21(DE3) or BL21(DE3)pLys. The over-expressed protein was enriched by the use of a nickle chelation column prepared from iminodiacetic acid sepharose. Fractions containing recombinant protein were pooled and loaded onto a Mono Q, HR 5/5 column (Pharmacia) operated by FPLC. The recombinant enzyme was eluted by a sodium gradient.

GST-CDPK$_\beta$, CDPK$_\gamma$. *E. coli* PR745 was the host cell used for the expression of GST-CDPK$_\beta$ and GST-CDPK$_\gamma$. The enzymes were purified by the

use of a glutathione agarose column and an anion exchange column—Mono Q (Lee et al., 1998).

Protein Analysis

Protein concentration was determined by the dye-binding method (Bradford, 1976) with a kit supplied by Bio-Rad Laboratories. Bovine plasma albumin (BSA) was used as the protein standard.

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out by the method of Laemmli (1970).

Protein Kinase Assay

Enzyme activity assay was performed by the modified procedure of Harmon et al. (1994). The assay buffer contained 50 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis(-aminoethyl ether)-N,N'-tetraacetic acid), 2 mM DTT, 0.1 mg/mL BSA, with or without 1.1 mM $CaCl_2$ for the presence or absence of calcium assay. The assay was performed in a total volume of 50 µL with 20 ng of recombinant CDPK, the indicated amount of peptide substrate and 60 µM [γ-^{32}P]ATP (500 cpm/pmol). Assay tubes were incubated for six minutes at 30°C before initiation of the reaction by addition of [γ-^{32}P]ATP. After 6 minutes, reactions were stopped by spotting 10 µL of the reaction mixture onto P81 paper. The paper squares were washed five times in 1% phosphate buffer, once in 95% ethanol, air-dried and counted in ScintiVerse BD in a liquid scintillation counter. For each of the CDPKs, at least three independent kinase assays were performed to determine their K_M and V_{max} to the substrate. The concentrations of the

substrate peptide used were 2-20 μM. Double reciprocal plots (1/v verses 1/[S]) were used for the determination of the kinetic parameters (K_M and V_{max}).

The activity of immobilized biotinylated $CDPK_\alpha$ was measured by a modification to the standard kinase assay. Affinity purified biotinylated $CDPK_\alpha$ was attached to a microtiter dish well coated with streptavidin. One hundred micromolar syntide-2 was used as the substrate, and the reaction was carried out at 24°C for six minutes.

Screening of the 15-Residue Random Peptide Phage Display Library

The random peptide library was obtained from Chiron Corporation through contract. The phage display random peptide library has about 2×10^7 random 15 amino acid sequences expressed on the surface of the phage M13 (Devlin et al., 1990). The library used had a titer of 1×10^{12} phage/mL and an insert frequency of about 55%.

The library screening relies on the strong biotin-streptavidin reaction to attach the interacting phage to a solid surface—usually polystyrene plates. The microtiter plate, Immulon 4 from Dayatech Laboratories. Inc., was used in this experiment. A microtiter dish well was coated with 4 μg streptavidin in 0.1 M $NaHCO_3$, pH 9.6 at 4°C overnight. The well was then blocked with blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 0.1 μg/mL streptavidin, 5 mg/mL BSA, 0.02% NaN_3) for two hours. After washing five times with TBSC/Tween buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM EGTA/0.5% Tween 20), 5.0 μg of biotinylated $CDPK_\alpha$ was added and incubated for two hours. The well was washed five times, 2 μL 10 mM biotin was added

and incubated for 15 minutes to saturate the remaining biotin-binding sites of streptavidin. Then 200 μL of random peptide library corresponding to about 1.0 $\times 10^{11}$ phages was added to the well and incubated for 4 hours. Phages on which the displayed peptides have bound to biotinylated $CDPK_{\alpha}$ were attached to the solid surface. The well was washed ten times with TBSC/Tween for one hour. Bound phages were eluted with 200 μL elution buffer (0.1 N HCl adjusted to pH 2.2 with glycine, 1 mg/mL BSA) for 10 minutes, immediately neutralizd with 12 μL 2 M Tris (pH unadjusted). The eluted phages were amplified as below and the amplified phages from the previous screening were used for the next one. The second and third rounds of screening were performed as in the fist round except that only 2.5 μg and 0.9 μg of biotinylated $CDPK_{\alpha}$ were used in each screening. The enrichment of affinity selection during the process was verified by PCR (polymerase chain reaction). The pair of primers were: 5'-CGATCTAA AGTTTTGTCGTCT-3'; 5'-AGCAAGCTGATAAACCG-3'.

Amplification of M13 Phage

The acid-eluted bacteriophages were amplified in *E. coli* JM109 cells. Briefly, the eluted phages were mixed with JM109 host cells and incubated at 37°C for 15 minutes, then mixed with 0.7% top agarose, poured onto the LB agar plate. After the plates had been incubated overnight, 6 mL LB medium was added to each plate, rocking slowly for 4 hours. The top medium containing diffused phage from the plate was collected and clarified by centrifugation at 13,000 g for 10 minutes. The supernatant was then mixed with 1/6 volume of PEG/NaCl (20% polyethylene glycol, 2.5 M NaCl). Bacteriophages were precipitated at 4°C for at least one hour or overnight. Precipitated phages were

collected by centrifugation at 13,000 g for 15 minutes. Pellet was suspended in TBSC (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM EGTA), re-precipitated with PEG/NaCl, the final precipitated phages were suspended in 200 µL TBSC with 0.02% NaN_3.

Purification of M13 Phage

A fresh phage plaque or one tenth of a suspension of the plaque was mixed with *E. coli* culture. The infected culture was incubated overnight in LB medium with 100 µg/mL ampicillin. The culture was centrifuged at 12,000 g for 5 minutes. The supernatant containing bacteriophage particles were mixed with 0.15 volume of PEG/NaCl to precipitate overnight at 4°C. Precipitated phages were collected by microcentrifugation for 15 minutes at 4°C. The pellet was dissolved in 1ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), re-precipitated with PEG/NaCl solution and dissolved in 0.15 M NaCl. One tenth of volume of 1.0 M acetic acid was added. After incubation for 10 minutes at room temperature and 10 minutes on ice, phages were collected by microcentrifugation at 4°C for 30 minutes. The pellet was suspended in 50 µL SM (100 mM NaCl, 10 mM $MgSO_4$, 20 mM Tris-HCl, pH 7.8). The titer of the final phage preparation was determined by the infection of a series of dilution of phages into *E. coli* cells.

Purification of M13 Single-Stranded DNA

The bacteriophage culture was obtained as described above. The culture was centrifuged at 12,000 g for 5 minutes. The supernatant containing bacteriophage particles were mixed with 1/6 volume of PEG/NaCl, standing for 15 minutes at room temperature. Precipitated phages were collected by

microcentrifugation for 15 minutes at 4°C. The pellet was suspended in 100 µL of TE by vigorous vortexing. 50 µL of phenol equilibrated with Tris-HCl (pH 8.0) was added. The tube was then vortexed for 30 seconds, standing for one minute at room temperature, vortexed again for another 30 seconds. The mixture was centrifuged for one minute and the upper, aqueous phase was transferred to a fresh microcentrifuge tube containing 300 µL of a 25: 1 mixture of absolute ethanol: 3 M sodium acetate (pH 5.2). The mixture was vortexed briefly and stored at room temperature for 15 minutes. The single-stranded M13 DNA was recovered by centrifugation at 12,000 g for 10 minutes at 4°C, washed by 70% ethanol. The pellet was air-dried and dissolved in 50 µL of TE. Purified single-stranded DNA was used as template for DNA sequencing.

Sequencing and Sequence Analysis

After three rounds of biopanning, eluted phages were plated at low density for the preparation of individual phage stocks. Single-stranded bacteriophage DNA was purified as above and sequenced by standard dideoxy termination DNA sequencing method (Sequenase Version 2.0, U. S. Biochemical Corp.) using primer (5′-CGATCTAAAGTTTTGTCGTCT-3′) near the random peptide cloning site. The nucleotide and polypeptide sequences were analyzed by the use of DNA Star (Madison, Wisconsin).

Binding Assay

The specificity of the binding of isolated phage to $CDPK_{\alpha}$ was determined by an independent binding assay. GST-$CDPK_{\alpha}$ attached to glutathione agarose beads was used for the assay. Phages were purified by the method described

above. Purified phages were incubated with 50 µL glutathione-agarose with GST-CDPK$_\alpha$ attached at 4°C for three hours. Then the agarose beads were washed ten times with TBSC/Tween buffer and suspended in 50 µL of TBSC/Tween. Three microliter of 0.1 unit/µL thrombin was added to the suspension and incubated for one hour. For control, no thrombin was added. The supernatant from thrombin cleavage was collected after centrifugation. The agarose beads were washed three times with TBSC/Tween. Solution from cleavage and wash step were combined and titered by infection into *E. coli* cells.

Synthesis and Analysis of the Peptide

The peptide corresponding to the insert sequence 6-3 of the specific binding phage plus surrounding amino acid residues (AERHPTLTRSPTLRNIQP PC) was synthesized at Protein Synthesis Lab at University of Florida. The concentration of the synthetic peptide was determined from amino acid composition analyses by Protein Synthesis Lab at University of Florida.

Construction of the 10-Residue Random Peptide Phage Display Library

Assembly of the double-stranded DNA insert from degenerated oligonucleotides

Two complementary oligonucleotides based on the sequence of peptide 6-3 from the first library screening were synthesized in DNA Synthesis Lab, University of Florida. The sequences are:

5'-CTT TCT ATT CTC ACT CCG CTG AAN NSN NSN NSN NSN NSN NSN NSN NSC GCC ACC CCA CGT TGA CGC GGT CGC CCA CGC TGC GGA ACA TCC AGC CGC CTC CAC CTC CAC C-3',

5′-**GGC CGG** TGG AGG TGG AGG CGG CTG GAT GTT CCG CAG CGT GGG CGA CCG CGT CAA CGT GGG GTG GCG SNN SNN SNN SNN SNN SNN SNN SNN SNN TTC AGC GGA GTG AGA ATA GAA AGG TAC-3′ (N=A+T+G+C, S=G+C). The underlined residues GGTACC are the *Kpn* I site. The residues in bold CGGCCG are the *Eag* I site.

An equal amount of each oligonucleotide (100 pmol) was mixed in 20 µL of annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl). The annealing mixture was heated at 95°C for 5 minutes, then cooled slowly in a water bath to room temperature. The annealed double-stranded DNA encodes peptides with 10 random amino acid residues located at the amino-terminal side of the peptide substrate 6-3. The peptide inserts were put at the amino terminus of the M13 coat protein III with AE at the very N-terminus of the mature protein as in the original library.

Preparation of linearized vector

The expression vector M13LP67 (Devlin et al., 1990) was modified for the construction of the new library to eliminate the growth of non-insertion clones. Briefly, double-stranded M13LP67 vector was digested with *Eag* I and *Kpn* I, followed by the calf intestinal alkaline phosphatase treatment. DNA from pBluescript SK was also digested by *Eag* I and *Kpn* I. The vector and insert fragments were gel-purified by QIAEX II gel extraction kit (QIAGEN Inc.). Two fragments were ligated and transformed into *E. coli* XL1-Blue cells. Positive clones were verified by restriction digestion. The new vector was named IL-1. Insertion of pBluescript SK fragment in IL-1 provided multiple stop codons in

the 5'-side of gIII, which would eliminate the growth of phages without insert sequences (vector IL-1 alone) in the amplification of the new library.

DNA of IL-1 was extracted with Wizard Plus Maxipreps (Promega) and further purified by equilibrium centrifugation in CsCl- Ethidium Bromide gradients (Sambrook et al., 1989). Purified IL-1 was digested with *Eag* I and *Kpn* I. The *Eag* I-*Kpn* I fragment was phenol extracted for the ligation reaction.

Ligation of the vector and insert DNA

Ten pmol of the double-stranded insert DNA and 1.0 pmol linear vector in a total volume of 200 µL were ligated by T4 DNA ligase (7 units) at 15°C overnight. The DNA was phenol extracted twice and dissolved in 5 µL TLE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

Electroporation of ligated DNA into *E. coli*

Ligated DNA (5 µL) was mixed with 100 µL ELECTROMAX DH12S™ cells (GIBCO BRL). The mixture was divided into five aliquots for five sets of electroporation to maximize the transformation efficiency. Electroporation was performed on Bio-Rad gene pulser. About 20 µL mixture was transferred into pre-chilled 0.1 cm cuvette which was placed into the sample chamber and the pulse was applied. The parameters for the electroporation were set to 1.7 kV, 25 µF, 200 Ω by the manufacturer's instruction. One milliliter of SOC medium (20 g/L Bacto-tryptone, 5.5 g/L Bacto-yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added immediately to the cuvette, and cultured for one hour at 37°C. The complexity of the library was

determined by plating serial dilution of the mixture of five electroporation aliquots for isolated plaques.

Amplification and harvest of phage library

The total 5 mL of electroporation solution was added to 200 mL 2xYT medium (16 g/L Bacto-tryptone, 10 g/L Bacto-yeast extract, 5 g/L NaCl) with 100 μg/mL ampicillin, shaking vigorously at 37°C overnight. The culture was centrifuged at 13,000 g for 10 minutes, supernatant containing the phage particles was PEG precipitated twice by the procedure described in "amplification of M13 library". The final precipitate was suspended in 5 mL of TBSC with 20% glycerol. The library was dispensed into 500 μL aliquots in sterile tubes, frozen by a dry ice: ethanol bath and stored at –80°C.

Screening of the 10-Residue Phage Display Library—Biopanning

The procedure for the screening of the 10-residue random peptide library was similar to that of 15-residue display library except for the following modifications. For the first round of biopanning, 100 ng of biotinylated $CDPK_{\alpha}$ was used. The enzyme was incubated with 1.0×10^{11} phages in TBSC/Tween at 4°C overnight. At the same time, a microtiter dish well was coated with streptavidin. The next day, the streptavidin-coated well was blocked with blocking solution and washed three times with TBSC/Tween. Then the phage-enzyme reaction mixture was transferred to the streptavidin-coated well and incubated for 10 minutes at room temperature. The well was washed 10 times with TBSC/Tween, eluted and amplified as in the first library screening. The

second and third rounds of biopanning were performed like the first one except that a lower amount of biotinylated $CDPK_{\alpha}$ was used—20 and 10 ng for the second and third round. After three rounds of biopanning, eluted phages were plated at low density and single plaques were isolated. The single-stranded DNA for each individual phage plaque was purified and sequenced by ICBR Sequencing Lab at University of Florida.

Phosphorylation Assay

To determine whether the displayed peptide of isolated phage were a substrate of CDPK, 250 ng of GST-$CDPK_{\alpha}$ and about 2×10^{10} phages were assayed by a modification of the protein kinase assay. After incubation for 10 minutes at 30°C, the reaction was stopped by adding 2× gel loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.05% bromophenol blue). The samples were boiled for 3 minutes, then loaded to the gel and separated by the SDS-PAGE. The radiolabeled gel was stained with Coomassie Blue, vacuum dried and exposed to a Kodak X-OMAT film.

Results

Identification of Five Peptides That Bound $CDPK_{\alpha}$ from the 15-Residue Library

Biotinylated $CDPK_{\alpha}$ was used for the random peptide library screening. The amount of biotinylated $CDPK_{\alpha}$ used in each round of screening was: 5 μg for the first round, 2.5 μg for the second round, and 0.9 μg for the third round. The same amount of phage (1.0×10^{11}) was used for each round of screening. The

number of phages eluted in each round increased from 1.0×10^4 (for the first round) to 3.5×10^6 (for the second round) and 2×10^9 (for the third round) in the first screening. This indicated that phages bound to the target protein were enriched along the screening. PCR was carried out using the eluted phage mixture as the template after each round of panning. Figure 2-1 shows that phages without random peptide inserts were eliminated just after the first round of screening. After three rounds of biopanning, eluted phages were plated at low density and 43 individual phage plaques were randomly selected. Single-stranded bacteriophage DNA was prepared and sequenced by standard dideoxy termination DNA sequencing method. The DNA sequence analysis of the isolated clones revealed that these phages displayed five different amino acid sequences (Table 2-1). Sequence 6-1, which was recovered with the highest frequency, was very rich in hydrophobic amino acids. Its high rate of recovery may have been due to the nonspecific binding to the matrix or binding to the calmodulin-like domain of CDPK. These peptide sequences are generally a

Table 2-1. Peptides isolated from the 15-residue random peptide library

Peptide	Sequences	Frequency
6-1	RPSLWRYWYQQPVYA	35
6-2	ASWFPTYFQQRFYTP	5
6-3	RHPTLTRSPTLRNIQ	2
6-4	WPFWYFPSKWKTFNQ	1
6-5	RLQPLKHHWEFFYQI	1

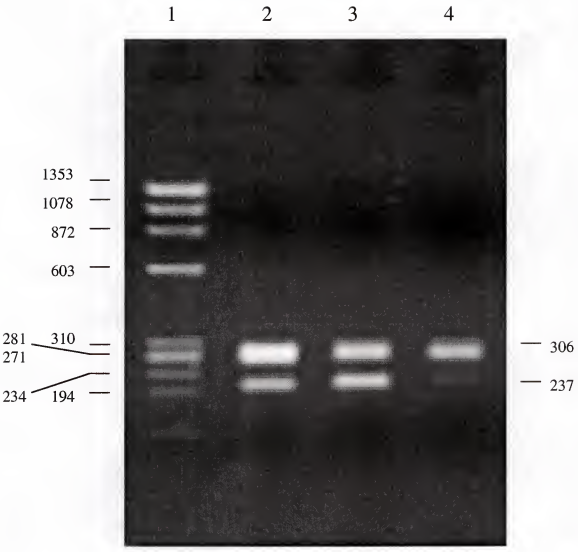


Figure 2-1. PCR analysis of 15-residue random peptide phage display library and the eluate from biopanning. PCR products were loaded to and resolved in a 2% agarose gel. Lane 1 is loaded with DNA molecular weight markers (øX174 DNA/*Hae* III Markers). Lane 2 is the PCR product of the original library. Lane 3 is the PCR product of the amplified library. Lane 4 is the PCR product of the eluate from the first round of biopanning. The band with the molecular weight of 306 base pair (bp) is the PCR fragment of phage with random peptide inserts. The band with the molecular weight of 237 bp is the PCR fragment of phage without random peptide inserts. The sizes of DNA molecular weight markers are indicated at the left side of the figure. From top to bottom, they are: 1353, 1078, 872, 603, 310, 281 and 271 (running at the same position), 234, and 194 bp.

combination of basic and hydrophobic residues, three out of five (sequence 6-2, 3, 4) contained the motif R/K-X-X-S/T (R/K = arginine/lysine, X = any amino acid, S/T = serine/threonine), which is the phosphorylation motif for CDPK.

One Peptide Bound $CDPK_\alpha$ Specifically in the Independent Binding Assay

To determine whether the phage from these isolates specifically bound to $CDPK_\alpha$, GST-$CDPK_\alpha$ attached to the glutathione agarose beads was used for the independent binding assay. Purified phage particles carrying insert peptide sequences 6-1 to 6-5 were incubated with agarose beads attached with GST-$CDPK_\alpha$. After a thorough wash with TBSC/Tween, thrombin was used to cleave GST-$CDPK_\alpha$. The purpose of this assay was to cleave GST-$CDPK_\alpha$, releasing only $CDPK_\alpha$ and the phage associated with it while leaving GST and nonspecifically bound phage on the agarose beads. Control assays were performed since phage particles could still be trapped in the agarose beads after extensive washing procedure. In control, purified phages were incubated with agarose beads attached with GST-$CDPK_\alpha$, but thrombin cleavage step was not carried out. Titers of phage particles released in the presence and absence of thrombin cleavage were compared. In the assay with phage No. 6-3, 10-fold more phages were released after thrombin cleavage over the control. This indicated the specific binding of peptide sequence 6-3 to $CDPK_\alpha$. Assays with the other four phage particles showed only 2 or 3-fold of phage released over control. Thus the peptide corresponding to the sequence of the insert peptide of phage 6-3 plus the surrounding amino acid residues (AERHPTLTRSPTLRNIQPPC) was synthesized for the further analysis.

Peptide 6-3 Was Phosphorylated by $CDPK_{\alpha}$

The synthetic peptide was assayed for the phosphorylation by GST-$CDPK_{\alpha}$, since it contained basis-X-X-Ser/Thr substrate motif of CDPKs. The peptide was phosphorylated by $CDPK_{\alpha}$ and was named CSP (CDPK substrate peptide). The stoichiometry of the CSP phosphorylation by GST-$CDPK_{\alpha}$ was measured by using 50 pmol of CSP and 400 ng of enzyme in the kinase assay. The calculated stoichiometry of the CSP phosphorylation is 1, indicating that there is one phosphorylation site per peptide molecule.

Kinetic Parameters for the Phosphorylation of CSP by Soybean CDPKs

The phosphorylation assays of CSP by GST-$CDPK_{\beta}$ and GST-$CDPK_{\gamma}$ were performed and both isoforms used CSP as the substrate. To examine the differences of three isoenzymes in the CSP phosphorylation, enzyme assays were performed with different concentration of CSP (2-20 μM). The kinetic parameters for the phosphorylation of CSP by three CDPKs were determined from the double reciprocal plots (Table 2-2). The apparent K_M and V_{max} of $CDPK_{\alpha}$, $CDPK_{\beta}$,

Table 2-2. Kinetic parameters for the phosphorylation of CSP in the presence of calcium by soybean CDPKs

CDPK Isoform	K_M (μM)	V_{max} (μmol/min/mg)	V_{max}/K_M
α	2.93	1.82	0.6
β	15.9	6.83	0.4
γ	1.23	0.31	0.25

and $CDPK_\gamma$ with CSP differed by as much as 13- and 22-fold, respectively. The ratio of V_{max} to K_M is an indicator of the enzyme catalytic efficiency with a substrate. Comparison of V_{max}/K_M for $CDPK_\alpha$, $CDPK_\beta$ and $CDPK_\gamma$ shows that $CDPK_\alpha$ had the greatest catalytic efficiency with CSP. Since $CDPK_\alpha$ was the enzyme used for the biopanning affinity selection, this result indicates that the peptide inserts specifically binding to the target protein can be isolated from the library screening.

Syntide-2 was the best peptide substrate for CDPKs before the finding of CSP, the apparent K_Ms of $CDPK_\alpha$, $CDPK_\beta$, and $CDPK_\gamma$ with syntide-2 are 18.2, 34.2 and 16.6 μM, V_{max}s of $CDPK_\alpha$, $CDPK_\beta$, and $CDPK_\gamma$ with syntide-2 are 2.4, 5.5 and 2.5 μmol/min/mg (Lee et al., 1998). The value of K_M for each CDPK with CSP was lower than that of syntide-2. The value of V_{max}/K_M was nearly the same for each CDPK with syntide-2 (0.13, 0.16, and 0.15). This indicates that syntide-2 is an equally good substrate for all three enzymes. The value of V_{max}/K_M for $CDPK_\alpha$, $CDPK_\beta$, or $CDPK_\gamma$ with CSP was higher than that for each CDPK with syntide-2, indicating that CSP is a better substrate than syntide-2.

Significant activity was observed for all three soybean CDPKs with CSP in the absence of Ca^{2+}. The kinetic parameters of CDPKs with CSP in the absence of

Table 2-3. Kinetic parameters for phosphorylation of CSP in the absence of calcium by soybean CDPKs

CDPK Isofrom	K_M (μM)	V_{max} (μmol/min/mg)	V_{max}/K_M
α	10.2	0.47	0.046
β	15.0	1.0	0.067
γ	4.67	0.03	0.006

calcium were determined (Table 2-3). The high basal level activity in the absence of calcium contributed to low values for activation by calcium (Table 2-4). $CDPK_{\alpha}$, $CDPK_{\beta}$, and $CDPK_{\gamma}$ were activated 25-, 50, and 100- fold by calcium respectively with syntide-2 as substrate.

Table 2-4. Phosphorylation of CSP in the presence and absence of Ca^{2+}

CDPK Isoform	V_{max} (μmol/min/mg)		Activation by Ca^{2+} (x-fold)
	$+Ca^{2+}$	$-Ca^{2+}$	
α	1.82	0.47	3.9
β	6.83	1.0	6.8
γ	0.31	0.03	10.3

Characteristics of Clones from the 10-Residue Random Peptide Library

In an effort to improve the binding affinity of the peptide, a new random peptide library was constructed. Based on the first library screening results, the new library extended the insert peptide sequence in length by adding 10 random amino acid residues to the amino-terminal side of the sequence and keeping peptide 6-3 sequence in place. The 10-residue library displayed peptides with the sequence: AE-X_{10}-RHPTLTRSPTLRNIQ-P_6 (X stands for any amino acid) followed by M13 coat protein III sequence. The new library contained about 1.0×10^7 different random 10 amino acid sequences displayed on the surfaces of M13 phage. The library was amplified and an aliquot of the library with about 1.0×10^{11} phages was used for the biopanning.

The biopanning procedure differed from that of 15-residue random peptide library in that phages were incubated with biotinylated $CDPK_{\alpha}$ first, the mixture of phages and $CDPK_{\alpha}$ was then added to the streptavidin coated microtiter dish well. Phages that had formed $CDPK_{\alpha}$-phage complex were immobilized onto the plate through biotin-streptavidin interaction.

After three rounds of biopanning, the eluted phages were plated at low density and 10 individual phage plaques were picked at random. The single-stranded DNA for each phage plaque was purified and sequenced by ICBR Sequencing Lab at University of Florida. The amino acid sequences of those clones are listed in table 2-5. There are 9 different peptide sequences encoded by

Table 2-5. Peptide isolated from the 10-residue random peptide library

Clone Number	Peptide Sequences	Frequency
7-1	TPWKNFTWFS	1
7-2	RWSPEVFWGW	1
7-3	VHWLREYWVG	1
7-4	TWNPFYNWAL	1
7-5	HAPVDSFAYT	1
7-6	GFVSPFSWYW	1
7-8, 11	VSPRSFWTTW	2
7-9	STFRSWFSYL	1
7-12	LSWGFPGWSW	1

10 $CDPK_{\alpha}$-binding phage isolates. These peptides are hydrophobic carrying aromatic amino acids or/and aliphatic amino acids. The motif #-X-X-X-X-#-# was deduced from the alignment of aromatic amino acids and aliphatic amino acids in the peptide sequences, # stands for aromatic and/or aliphatic amino acids and X stands for any amino acid residue (Figure 2-2). These hydrophobic residues may be responsible for the additional interactions between the displayed peptide and $CDPK_{\alpha}$.

To test the characteristics of these displayed peptides, the protein phosphorylation assay was performed with purified phages and GST-$CDPK_{\alpha}$ (Figure 2-3). Two controls of purified plain M13 phage with vector alone and M13 phage with displayed peptide 6-3 were included. About 5.0×10^9 phages were used in the assay for each isolate. Except for the vector-only control, all the sample lanes had a phosphorylation band corresponding to the M13 pIII fusion protein with the displayed peptide, indicating that all the displayed peptides of isolated phage clones were the substrates of $CDPK_{\alpha}$. Since sequence No. 7-8,11 occurred twice and its peptide sequence agreed well with the consensus alignment, this sequence together with the peptide 6-3 sequence was selected and cloned into a binary vector pBI121 for the transformation of tobacco leaf discs and later *in vivo* studies. The phage displaying peptide 7-8,11 plus 6-3 sequence was named as phage 2-97-8.

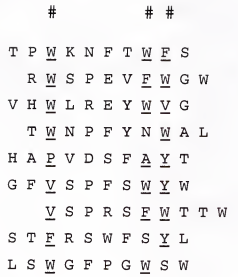
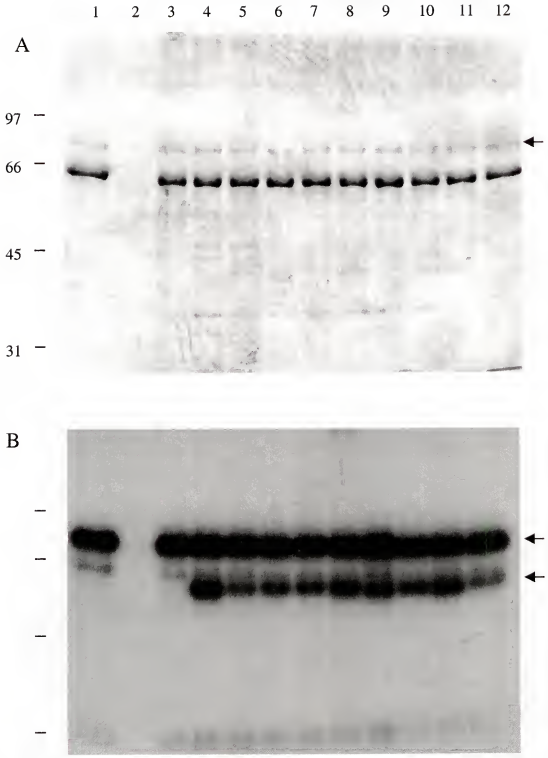


Figure 2-2. Alignment of nine peptide sequences from 10-residue random peptide phage display library biopanning. The sequences are arranged by their alignment of aromatic amino acids and aliphatic amino acids, which are underlined in individual amino acid sequence and indicated by the symbol of # at the top of the alignment. The motif #-X-X-X-X-#-# was deduced from this alignment, X stands for any amino acid residue. The order of the sequences is corresponding to that of table 2-5.

Figure 2-3. Phosphorylation of phage displayed peptides by GST-CDPK$_\alpha$. Individual phage clones from 10-residue random peptide phage display library were purified. The purified phage were used in the phosphorylation reaction with GST-CDPK$_\alpha$, which is stopped by the addition of gel-loading buffer and subjected to electrophoresis in 10% SDS-polyacrylamide gel. After staining with Coomassie Blue, the gel was dried and exposed to an X-ray film. Panel A: Coomassie-stained gel, the arrow indicates the position of GST-CDPK$_\alpha$. The heavily stained band cross the gel is bovine serum albumin used in the buffer. Panel B: autoradiograph of the same gel as in the panel A, the top arrow indicates autophosphorylated GST-CDPK$_\alpha$; the bottom arrow indicates phosphorylated pIII fusion protein with displayed peptides. In panel A and panel B, lane 1 GST-CDPK$_\alpha$ alone control; lane 2 phage without peptide insert alone control; lane 3 phage without random peptide insert; lane 4 phage 6-3 (with displayed peptide 6-3); lane 5 phage clone No. 7-1; lane 6 phage clone No. 7-2; lane 7 phage clone No. 7-3; lane 8 phage clone No. 7-4; lane 9 phage clone No. 7-5; lane 10 phage clone No. 7-6; lane 11 phage clone No. 7-8,11; lane 12 phage clone No. 7-9. The positions of protein molecular weight markers are indicated by the lines at the left side of each panel. From top to bottom, they are: 97, 66, 45, and 31 kD.


1 2 3 4 5 6 7 8 9 10 11 12
A
97
66
45
31
B

Discussion

Optimization of Conditions for Phage Display Library Screening

Preparation of the active immobilizable enzyme

The library screening procedure required that CDPK be immobilized onto a solid surface to facilitate the isolation of phages whose displayed peptides interact with the enzyme.

First, the chemical crosslinking of biotin to CDPK was attempted in order to achieve the immobilization through biotin-streptavidin interaction. His_6-$CDPK_\alpha$ was biotinylated with Biotin-X-NHS (Calbiochem) according to the method of Billingsley et al. (1985). Biotinylated $CDPK_\alpha$ was detected by ExtrAvidin-alkaline phosphatase conjugate (Sigma). But the kinase activity of biotinylated His_6-$CDPK_\alpha$ was lost totally. The possible reason is that biotin maybe crosslinked to the lysine residue in His_6-$CDPK_\alpha$ that is an important residue for the binding of ATP to the active site of the kinase, as a result biotinylated His_6-$CDPK_\alpha$ lost the kinase activity.

Another immobilization method using the crosslinking between the primary amines was attempted. His_6-$CDPK_\alpha$ was coupled to AminoLink™ Gel (Pierce) according to the manufacturer's instruction. The coupled enzyme was partially active (data not shown) and the column was used for the initial screening of the phage display library. Because of problems encountered with an agarose column (see Procedures for the random peptide library screening), this method was not pursued.

PinPoint™ Xa protein purification system (Promega) was used to obtain the immobilizable, active $CDPK_{\alpha}$. $CDPK_{\alpha}$ cDNA was cloned into a PinPoint™ Xa-3 vector downstream of a sequence encoding a subunit of the transcarboxylase complex from *Proprionibacterium shermanii* that becomes biotinylated *in vivo* when the fusion protein is expressed in *E. coli*. The immobilized biotinylated $CDPK_{\alpha}$ was active and the activity was calcium-dependent. The biotinylated $CDPK_{\alpha}$ was used as the bait for the random peptide library screening.

Procedures for the random peptide library screening

His_6-$CDPK_{\alpha}$ AminoLink gel column was used for the initial screening. Screening was performed in a manner similar to protein chromatography. The column was loaded with an aliquot of the random peptide library, then washed with buffer and eluted with acid. The eluted phages were amplified and the affinity chromatography was performed twice more. Individual plaques were selected and analyzed by PCR. Surprisingly only 20% of the clones picked contained peptide inserts. This meant that the affinity chromatography didn't enrich the phages with displayed peptides specifically interacting with $CDPK_{\alpha}$. The possible reasons are the use of the agarose column and elution condition. Phage applied to the column could be trapped nonspecifically in the agarose gel even though an extensive washing procedure was performed, the trapped phage could be released in the subsequent acid elution. The affinity chromatography was not pursued since the inherent problems with the column screening.

A microtiter dish plate was used as the matrix to capture the biotinylated $CDPK_{\alpha}$. Different approaches were used for the library screening. For the 15-residue random peptide library, an aliquot of the library was added to the microtiter dish well coated with biotinylated $CDPK_{\alpha}$ and incubated for hours to allow the binding of displayed peptides to immobilized $CDPK_{\alpha}$. For the 10-residue random peptide library screening, an aliquot of the library was incubated with biotinylated $CDPK_{\alpha}$ overnight, then the reaction mixture was added to the microtiter dish well coated with streptavidin and incubated for 10 minutes to allow the preformed phage-$CDPK_{\alpha}$ complex attached to the plate. In this way, the interaction between the displayed peptides and enzyme occurred in solution, the amount of enzyme used could be easily adjusted so that the mole ratio of enzyme and phage displayed peptides could be manipulated to introduce the competition in the binding of phage to $CDPK_{\alpha}$.

Construction of the 10-Residue Random Peptide Phage Display Library

To facilitate the use of peptide substrates in later studies, the affinity between the substrate and CDPK needed to be improved. PKI (5-24), corresponding to residues 5-24 of an endogenous protein kinase inhibitor (PKI) of Protein kinase A (PKA), inhibits PKA with almost the same potency (K_i = 2.3 nM) as the full length protein (Cheng et al., 1985). The peptide contains a pseudosubstrate site that mimics the substrate motif of PKA. X-ray crystallographic studies confirmed previous biochemical evidence that PKI (5-24) binds to the active site of PKA (Knighton et al., 1991). Studies of the mechanism of regulation of CDPK (Harmon et al., 1994; Harper et al., 1994) and other calcium-regulated protein kinases (Cruzalegui et al., 1992) have indicated that

these enzymes contain internal pseudosubstrate sites that bind to the active site in the absence of calcium. Synthetic peptides analogous in sequence to the autoinhibitory domain of soybean $CDPK_\alpha$ inhibited the enzyme with a K_i of 2 μM (Harmon et al., 1994). The N-terminal residues from the pseudophosphorylation site contribute greatly to the peptide-enzyme interaction (Kemp et al., 1994). The optimal lengths of peptide inhibitors of PKA and myosin light chain kinase are 20 residues (Kemp et al., 1991). It is possible to have extra amino acid residues at the amino end of CSP to elongate the peptide substrate to achieve a higher affinity interaction. The new library based on the result of the 15-residue random peptide library screening was constructed. In the new random peptide library, ten additional random amino acids were inserted to the amino-terminal side of clone 6-3 sequence. The library displays peptide AE-X_{10}-RHPTLTRSPTLRNIQ-P_6 (X stands for any amino acid) which is fused with M13 coat protein III.

Characterization of CSP

The peptide corresponding to peptide sequence 6-3 plus surrounding amino acid residues was synthesized and tested to be a good substrate of CDPK. CSP has two possible phosphorylation sites: AERHP**T**LTRSP**T**LRNIQPPC, substrate motifs are underlined, the potential phosphorylation sites are in bold. But the stoichiometry of CSP phosphorylation by $CDPK_\alpha$ was measured to be 1, which indicates there is only one phosphorylation site per peptide molecule. Compared with sequences of other CDPK peptide substrates (Figure 2-4), the threonine in the RSPT was predicted to be the phosphorylation site. Similar to other preferred peptide substrates of $CDPK_\alpha$, CSP has leucine carboxyl-terminal

		-5	-3				P	+1			+4		
	Motif	L	X	R	X	X	S/T	V	X	X	L		
Syntide-2	P	L	A	R	T	L	S	V	A	G	L	PGKK	
Autocamtide-2	KKA	L	R	R	Q	E	T	V	D	A	L		
MLCKsk	AKR	P	Q	R	A	T	S	N	V	F	S		
MLCKsm	KKR	A	A	R	A	T	S	N	V	F	A		
CSP	**AERHPT**	**L**	**T**	**R**	**S**	**P**	**T**	**L**	**R**	**N**	**I**	**QPPC**	
CSP		A	E	R	H	P	T	L	T	R	S	PTLRNIQPPC	

Figure 2-4. Peptide substrates of CDPK. The sequences of peptide substrates are shown with the phosphorylated amino acids (position P) aligned. The consensus phosphorylation motif $LXRXX^{S}/_{T}VXXL$ is shown with residues numbered relative to the phosphorylated residue marked by P. This motif was deduced to be preferred by $CDPK_{\alpha}$ by comparison of the sequences of two good substrates (syntide-2 and autocamtide-2) and two poor substrates (skeletal and smooth muscle myosin light chain kinase substrate peptides—MLCKsk and MLCKsm, respectively) (Lee et al., 1998). Two possible alignments of CSP are shown, and the alignment that better matches the consensus motif is in bold.

next to it, isoleucine at four residues carboxyl-terminal and leucine five residues amino-terminal from it. A previous report also indicated that peptides with aliphatic amino acid residues present in those positions are preferred substrates of $CDPK_{\alpha}$ (Lee et al., 1998).

The identification of peptide 6-3—the best peptide substrate of CDPK from the 15-residue random peptide phage display library demonstrated that affinity purification (biopanning) could select the specific interaction peptides of target proteins. Sequence analysis showed that CSP had the basic-X-X-Ser/Thr general substrate motif of CDPK and it also carried aliphatic amino acids at certain positions that is consistent with the consensus sequences for the substrates of $CDPK_{\alpha}$ deduced from other biochemical data. A 10-residue random peptide phage display library was constructed based on the peptide 6-3 sequence. Sequences isolated from 10-residue library had the #-X-X-X-X-#-# (# = hydrophobic amino acid) consensus alignment. One peptide substrate sequence 2-97-8 obtained from two library screenings was used for the transformation of tobacco leaf discs described in following chapter.

CHAPTER 3
PHOSPHORYLATION STUDIES OF CDPK PEPTIDE SUBSTRATE IN TRANSGENIC TOBACCO CELLS

Introduction

Soybean CDPK is a member of a large family of protein kinases that contain three functional domains: a catalytic domain and a calmodulin-like domain that are separated by an autoinhibitory domain (Roberts and Harmon, 1992). Since CDPK has its own regulatory domain (calmodulin-like domain), it is activated by elevated calcium alone (Huang et al., 1996; Yoo and Harmon, 1996). Changes in the intracellular free calcium concentration have been connected to many signal transduction pathways in response to various stimuli or stresses, such as drought, light, cold, mechanical stimulation, and pathogen attack.

Calcium-regulated protein kinases are involved in amplifying and diversifying the action of Ca^{2+} mediated responses. In plants, calcium-dependent, calmodulin-independent CDPKs seem to be the predominant calcium-dependent protein kinases. CDPKs have been purified and cloned from soybean and many other plants, but the physiological roles of CDPKs remain unclear. Because of the easy manipulation of the tobacco tissue and cell culture, tobacco plants and tobacco suspension cultures have been used in the research of calcium involved signal transduction. Tobacco seedlings that were genetically transformed to express apoaequorin were incubated in *h*-coelenterazine to

reconstitute the calcium-sensitive luminescent protein aequorin. Touch, cold shock, elicitors, and wind caused increased cytosolic calcium (Knight et al., 1991; Knight et al., 1992). Hydrogen peroxide treatment of these seedlings resulted in a transient increase of calcium which lasted several minutes (Price et al., 1994). Suspension cultured tobacco cells that express aequorin were demonstrated to respond to cold shock as do the intact transgenic plants from which they were derived. Oligogalacturonic acid (OGA), Mas-7 (a peptide known to activate G protein and Ca^{2+} fluxes), and hypoosmotic stress triggered a rapid increase in cytosolic Ca^{2+} (Chandra et al., 1997). Using tobacco suspension culture cells, Takahashi et al. observed that hypoosmotic shock and mastoparan induced an increase in cytosolic Ca^{2+} concentration and subsequent activation of protein kinases (Takahashi et al., 1997; Takahashi et al., 1998). The involvement of calcium in the hypersensitive reaction of tobacco cells to cryptogein, a proteinaceous elicitor was confirmed by inhibitor studies (Tavernier et al., 1995).

Transient increases in intracellular calcium have been observed in plant cells under many conditions (Gilroy and Trewavas, 1994). These observations have stimulated research on calcium-regulated protein phosphorylation in plants. The evidence for calcium-dependent protein phosphorylation in response to different stimuli has accumulated. Hypoosmotic signal induced protein phosphorylation following cytosolic Ca^{2+} elevation in tobacco suspension culture cells (Takahashi et al., 1997), a very rapid red light-induced calcium-sensitive protein phosphorylation was observed in etiolated wheat leaf protoplasts (Fallon et al., 1993).

Calcium and protein kinases have also been implicated as early response elements in plant disease resistance. In parsley cells (*Petroselinum crispum*),

fungal elicitor stimulates ion fluxes, especially activates a calcium-permeable ion channel and causes the production of reactive oxygen species (ROS), defense gene activation and phytoalexin accumulation (Jabs et al., 1997; Zimmermann et al., 1997). In tomato, activation of one plasma membrane Ca^{2+}-permeable channel was induced by race-specific fungal elicitors (Gelli et al., 1997). Protein phosphorylations are essential for the oxidative burst elicited by oligogalacturonic acid in cultured soybean suspension cells (Chandra and Low, 1995). A calcium-dependent protein phosphorylation is involved in the activation of NADPH oxidase upon treatment of elicitor-containing intercellular fluids in tomato cells (Xing et al., 1997).

Plant disease resistance to pathogens often depends on whether the plant is able to recognize the pathogen early in the infection process. The recognition event leads to a rapid tissue necrosis at the site of infection, which is called the hypersensitive response (HR). One of the most striking early events of HR is a rapid release of reactive oxygen species, which is termed the oxidative burst. ROS are toxic intermediates that result from successive one-electron reaction of O_2 reduction. The oxidative burst leads to the generation of superoxide and subsequent accumulation of H_2O_2. Hydrogen peroxide from the oxidative burst not only drives crosslinking of cell wall structural proteins, but also acts as a local trigger of programmed cell death in challenged cells and as a diffusible signal for the induction of genes encoding cellular protectants such as glutathione S-transferase (GST) in adjacent cells (Levine et al., 1994; Tenhaken et al., 1995). However, little is known about the molecular events downstream of oxidative signals. There is also evidence that oxidative signals stimulate an increase in cytosolic calcium. Hydrogen peroxide stimulated transient calcium-

dependent luminescence was observed in apoaequorin transformed tobacco seedlings (Price et al., 1994). Hydrogen peroxide also induced a transient cytosolic Ca^{2+} elevation in tobacco suspension cells (Takahashi et al., 1998). In soybean cells, H_2O_2 stimulates a rapid influx of Ca^{2+} into the cells, which activates a physiological cell death program (Levine et al., 1996). Guard cell cytosolic free calcium increases in response to oxidative stress in *Commelina communis*, which results in the inhibition of stomatal opening and promotion of stomatal closure (McAinsh et al., 1996).

Glutathione (GSH) is an essential metabolite and a powerful regulator of major cell functions (Alscher, 1989; Kreuz et al., 1996; Marrs, 1996; Steffens, 1990). GSH and GSH-dependent processes appear to play a key role in plant-pathogen interactions (Foyer et al., 1997; May et al., 1998). The cloning of serine acetyltransferase (SAT) from interaction cloning with soybean CDPK connected CDPK to plant defense responses. SAT is one of the two enzymes catalyzing the biosynthesis of cysteine. Phosphorylation of SAT *in vitro* by CDPK relieves the feedback inhibition of SAT by cysteine (B.-C. Yoo and A. C. Harmon, personal communication). Concentrations of cysteine are kept low in the cells, owing to the reactivity of the thiol group. It is proposed that cysteine-insensitive SAT can produce more O-acetyl-serine that leads to the synthesis of extra cysteine. Cysteine is rapidly converted to the tripeptide glutathione (GSH): glutamate-cysteine-glycine. GSH is then used as an antioxidant to protect cells surrounding the site of localized necrosis from oxidative damage caused by pathogen invasion. Consistent with this, increases in the levels of GSH have been measured in bean and alfalfa suspension cells treated with fungal elicitors

(Edwards et al., 1991), and H_2O_2 from an oxidative burst was shown to stimulate GSH synthesis (May et al., 1996; May and Leaver, 1993).

It is important to determine whether CDPK is involved in plant defense response and when CDPK is activated *in vivo*. The purpose of the present study is to address these questions by the use of transgenic tobacco suspension cells that express a CDPK substrate. The strategy of introduction of exogenous substrates has been successfully used in defining the timing of kinase activities during mitotic progression in *Tradescantia virginiana* stamen hairs (Wolniak and Larsen, 1995), or in highlighting the involvement of a kinase that specifically phosphorylates myosin light-chain kinase in flagellar activity (Ashizama et al., 1995). By microinjection of the peptide substrate syntide-2, Ritchie and Gilroy identified a calcium-dependent protein kinase involved in the GA response in barley aleurone protoplasts (Ritchie and Gilroy, 1998).

In the present study, a high affinity peptide substrate was obtained from the screening of random peptide phage display libraries (described in Chapter 2). The substrate peptide sequence together with the double hemagglutinin (HA) epitope sequence was cloned into a plant expression vector and transformed into tobacco. The transgenic suspension culture was derived from transformed tobacco calli and was used for the experiments. The expressed peptide substrate was phosphorylated *in vitro* and *in vivo* in tobacco cells. Application of hydrogen peroxide to the transgenic tobacco cells caused increased phosphorylation of the CDPK peptide substrate.

Material and Methods

Plant Material

Axenic tobacco plants (*Nicotiana tabacum* cv. Wisconsin 38, obtained from Dr. Mike Niuccio, University of Florida) were subcultured on TSM medium in Magenta boxes at 24°C. TSM medium contained MS salts, MS vitamins, 3% sucrose, 0.05 mg/L kinetin, 0.1 mg/L IAA (indoleacetic acid), 0.8% (w/v) agar.

Plasmid Construction

The substrate peptide sequence of 2-97-8 was cloned into a plant expression vector pBI121 (Clontech). A double HA (hemagglutinin) epitope tag was inserted between the substrate peptide and β-glucuronidase (GUS) for the convenience of immunoprecipitation.

Peptide substrate. In order to create restriction sites for the cloning, PCR was performed using single-stranded DNA of phage 2-97-8 as template. The pair of primers were:

5'-primer, 5'-GCACGTCTAGA**ATG**TCTCACTCCGCTGAA-3';

3'-primer, 5'-CGTGAGGTACCCGGTGGAGGTGGAGG-3'.

Underlined residues TCTAGA are the *Xba* I site. Underlined residues GGTACC are the *Kpn* I site. ATG in bold is the added translation initiator codon. Twenty-eight cycles of PCR was performed using the following temperature profile: denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, primer extension at 72°C for 1 minute. Cycling was initiated with first denaturation at 94°C for 5 minutes and concluded with a final extension at 72°C for 5 minutes. The PCR product was purified by phenol extraction and digested with *Xba*I and

*Kpn*I. The digested PCR product was purified by phenol extraction. The *Xba*I-*Kpn*I fragment encoded the substrate peptide sequence as well as had an ATG translation initiation codon at the 5' end.

HAHA fragment. Two complementary oligonucleotides based on the sequence of the double HA epitope tag (YPYDVPDYA) with glycine (G) in between were synthesized by GIBCO BRL.

The forward strand sequence is: 5'-**CT**ACCCATACGACGTTCCAGACTACGCT GGTTACCCATACGACGTTCCAGACTACGCCCC-3'.

The reverse strand sequence is: 5'-GGGGCGTAGTCTGGAACGTCGTATGGGT AACCAGCGTAGTCTGGAACGTCGTATGGGTA**GGTAC**-3'.

Underlined residues CCCGGG are the *Sma* I site. Residues in bold GGTACC are the *Kpn* I site.

Equal amounts of each oligonucleotide were added to the annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl). The mixture was heated at 95°C for 5 minutes, then cooled slowly in a water bath to room temperature.

Linearized vector. The vector pBI121 DNA was digested with *Sma*I and *Xba*I. The digested vector was phenol extracted.

Ligation and transformation of IpBI121. Three nucleotide fragments: linearized vector, peptide substrate fragment and HAHA fragment (in a ratio of 0.1pmol: 1pmol: 1pmol) were mixed and ligated with T4 DNA ligase (1 unit) at 15°C overnight. The new construct encoded a fusion protein that has the substrate peptide at the amino terminus, double HA eitope tag in between with a six amino acid linker of PGGQSL followed by the in-frame GUS product. The ligation product was transformed into *E. coli* DH5α. Positive clones were

verified by restriction enzyme digestion analysis. The DNA sequence of the positive clone was confirmed by DNA sequencing (ICBR DNA Sequencing Lab at University of Florida) using GUS DNA sequencing primer 5'-TCACGGGTTG GGGTTTCTAC-3'. The primer located downstream of the ATG initiation codon of the sequence of GUS gene in vector pBI121. The resulting plasmid, pBI121-phag (with peptide substrate, HAHA, and GUS in order), was transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986) by the freeze-thaw method (An et al., 1988).

Agrobaterium-Mediated Transformation

Tobacco was transformed with pBI121-phag by the leaf disc transformation method (Horsch et al., 1985) with some modifications. About 4 mm-wide leaf strips were cut from aseptic tobacco leaves, and immediately submerged in a diluted culture of *A. tumefaciens* grown overnight in YEP medium (10 g/L yeast extract, 10 g/L Bacto-peptone, 5 g/L NaCl) at 28°C. Leaf strips were incubated for 20 minutes with gentle shaking to ensure that all the edges were inoculated. Then the leaf strips were blotted dry and incubated for 2 days on callus-induction medium (CIM) which contained MS salts, B5 vitamins, 30 g/L sucrose, 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8% agar at 25°C in the dark. After cocultivation, leaf strips were transferred to selection medium (CIM with 100 µg/mL kanamycin, 50 µg/mL Timentin). Leaf strips were transferred to fresh selection medium every 5 days for 15 days and weekly thereafter.

Establishment of the Cell Suspension Culture

When calli were well formed around the edges of leaf strips, tobacco cell suspension cultures were initiated by dispensing friable and soft calli to BY-2 medium containing 100 µg/mL kanamycin, 50 µg/mL Timentin and 0.2 mg/L 2,4-D. Timentin was used in the first month for complete inhibition of the growth of *Agrobacterium* that might be present in the suspension culture. Kanamycin was included throughout these experiments to maintain selection for transgenic cells. BY-2 medium was modified from Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) in which the level of KH_2PO_4 and thiamine-HCl was increased to 370 mg/L and 1 mg/L (Shimizu et al., 1996). Suspended calli were allowed to grow for two to three weeks to achieve the formation of the suspension. After the tobacco cell suspension was established, the tobacco cell culture was maintained by transferring 8 ml of cell suspension in 72 ml of fresh medium once every week.

Tobacco Cell Extraction

For all assays and immunoprecipitation procedures, tobacco suspension cells that had been subcultured to fresh medium three days previously were used for the extraction. Tobacco cells were homogenized in extraction buffer that was also used for immunoprecipitation (IP). IP buffer contained 50 mM Hepes, pH 7.2, 50 mM NaCl, 2 mM EDTA, 2 mM DTT, 5% glycerol with 1 mM EGTA, protein kinase inhibitor (1 µM Staurosporine), phosphatase inhibitors (10 mM NaF, 1 µM Microcystin-LR), and protease inhibitors (20 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM PMSF). Cells were extracted in a ratio of 1 mL

extraction buffer per gram of cells. Homogenate was centrifuged at 12,000 g for 10 minutes. The supernatant was transferred to a fresh centrifuge tube, and was centrifuged at 40,000 g for an hour to pellet all the membrane fractions. Then the supernatant was used for the experiment or enzyme assay.

Immunoprecipitation

Three-day-old tobacco suspension cells were homogenized in the same volume of IP buffer. Homogenate was centrifuged as described above and the supernatant was used for immunoprecipitation. The supernatant was incubated with 10 µL Pansorbin cells (Calbiochem) for 30 minutes at 4°C to eliminate plant proteins that non-specifically bind to Pansorbin cells. The supernatant was incubated with 1 µg anti-HA (Boehringer Mannheim) for one hour at 4°C. Then 10 µL Pansorbin cells or protein G beads (Sigma) were added to the mixture and incubated for 30 minutes (protein G beads were used later to reduce the nonspecific binding of Pansorbin cells to the tobacco cell extract). Pansorbin cells/protein G beads with bound antibody-antigen complexes were centrifuged and washed twice with IP buffer and suspended in the indicated buffer.

The phosphorylation reaction of the immunoprecipitated PHAG by $CDPK_{\alpha}$ was carried out according to the protein kinase assay method described in Chapter 2 except that immunoprecipitated PHAG was used instead of the synthetic peptide substrate. Since GST-$CDPK_{\alpha}$ and the expressed phag-construct protein have similar molecular weights, autophosphorylated CDPK band and phosphorylated PHAG band were hardly distinguishable in the SDS-PAGE gel and autoradiograph, and this interfered with the interpretation of the data. To remedy this problem, the lower molecular weight His_6-$CDPK_{\alpha}$ was used later for

the phosphorylation assay. Immunoprecipitated PHAG was suspended in assay buffer. The reaction was stopped by adding EGTA to 10 mM and immediately transferred onto ice. The microtube containing the reaction mixture was centrifuged, and supernatant was saved. Pansorbin cells/protein G beads with PHAG-anti-HA antibody complex attached were washed twice with IP buffer and resuspended in 1× loading buffer. Proteins were separated by SDS-PAGE, then stained with Coomassie Blue or electroblotted onto a nitrocellulose membrane.

Immunoblot

Proteins were electroblotted onto a nitrocellulose membrane (Micron Separation Inc.). The membrane was stained with Ponceau-S and the image was scanned. The membrane was covered with blocking buffer (5% nonfat dry milk in 1×TBS) for one hour after it had been destained in 1×TBS (20 mM Tris-HCL, pH 7.5, 500 mM NaCl). Then the membrane was incubated with anti-HA—0.25 μg/mL in antibody dilution buffer (1% gelatin in TBS with 0.02% NaN_3) for one hour, washed with 1×TTBS (1×TBS with 0.05%v/v Tween-20) twice for 10 minutes. The membrane was covered with anti-mouse IgG alkaline phosphatase conjugate for one hour, washed as before. The membrane was then incubated in color development solution (0.1 mg/mL nitro blue tetrazolium and 0.05 mg/mL BCIP (5-bromo-4-chloro-3-indoyl phosphate) in 0.15 M $NaHCO_3$, pH 9.6, 4.0 mM $MgCl_2$) for 10-60 minutes. The membrane was washed in water, dried and if phosphorylation assays or *in vivo* labelings had been performed, the immunoblotted membrane was exposed to an X-ray film.

GUS Assays

GUS assays were performed using the methods described by Jefferson (1987).

Histochemical staining. Calli or suspension cells were stained in X-glucuronide (X-gluc) solution (1.0 mM X-gluc in 0.1 M $NaPO_4$ buffer, pH 7.0, 10 mM EDTA, 0.5 mM KFerricyanide, 0.5 mM KFerrocyanide, 0.1% Triton X-100) at 37°C for 10 minutes to one hour. Calli and suspension cells from pBI121-phag transformed tobacco, untransformed tobacco cells, and vector pBI121 alone transformed cells were used for staining.

Fluorometric assay. Three-day-old tobacco suspension cells were collected and homogenized in the same volume of extraction buffer (0.1 M sodium phosphate, pH 7.0, 2 mM EDTA, 2 mM DTT, 5% glycerol) with protease inhibitors (20 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM PMSF). The homogenate was centrifuged and supernatant was transferred to a fresh tube for the assay. For GUS activity measurement of immunoprecipitated-PHAG, Pansorbin cells or protein G agarose beads with immunoprecipitated proteins were suspended in the extraction buffer. The fluorogenic reaction was carried out in 1 mM MUG (4-methylumbellifery β-D-glucuronide). The reaction was performed at 37°C, 50 µL of reaction mixture was removed at each time point, the reaction was terminated with 0.95 mL 0.2 M Na_2CO_3. Fluorescence was then measured with excitation at 365 nm, emission at 455 nm on a spectrofluorometer. The fluorometer was calibrated with freshly prepared MU (4-methylumbelliferone) standards in the same reaction buffer.

In vivo Labeling and Hydrogen Peroxide Treatment

Three-day-old tobacco suspension cells were suspended in labeling buffer and agitated at 60 rpm. Labeling buffer contained 0.85 mg/L KH_2PO_4, 1.09 mg/L K_2HPO_4, 18.7 mg/L KCl, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 27.7 mg/L $CaCl_2$, 8.7 mg/L ferric monosodium EDTA, 250 mg/L MES, pH 5.7, and 30 g/L sucrose. Two hundred and fifty microcuries of phosphorus-32 radionuclide (H_3PO_4, 10 mCi/ml, DuPont NEN) was added to 5 mL suspension cells. Cells were labeled for one hour before any treatments. Control cells agitated at 60 rpm were labeled without treatment for two hours. Hydrogen peroxide was added to the suspension culture at the final concentration of 2 mM and cells were labeled for one more hour. After the labeling or treatment, tobacco cells were quickly drained, washed, and frozen with liquid nitrogen. Frozen cells were extracted as described before.

Quantitative Analysis of Immunoblots and Autoradiographs

Quantitative analysis of the immunoblots and autoradiographs was performed on a Macintosh computer using the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Blots and autoradiographs were calibrated to known standards.

Results

Construction of pBI121-Phag Fusion Protein Expression Vector

For the expression of foreign genes in planta, a vector pBI121 with cauliflower mosaic virus (CaMV) 35S promoter was chosen to construct the expression vector. The peptide substrate sequence of 2-97-8 and the double HA epitope tag sequence were cloned into the binary vector plasmid pBI121, thus giving rise to plasmid pBI121-phag. Figure 3-1 shows the structure of the constructed fusion gene. In pBI121-phag, an extra ATG was added to 5' end of the peptide substrate sequence to introduce an initiation codon. The cloned fragment encodes a fusion protein starting with Met, followed by the peptide substrate and adjacent residues in the phage sequence (SHSAEVSPRSFWTTWR HPTLTRSPTLRNIQ P_6GT), double HA epitope tag sequence (YPYDVPDYAGYP YDVPDYA) followed by PGGQSL, and β-glucuronidase from amino-terminus to carboxy-terminus.

Agrobacterium-Mediated Transformation and Tobacco Calli Induction

Plasmid pBI121-phag was transformed into tobacco (Wisconsin 38) by *Agrobacterium*-mediated transformation. As control, the vector plasmid pBI121 was introduced into tobacco by *Agrobacterium*-mediated transformation as well. The transformed tobacco leaf strips were selected on kanamycin/timentin plates. One week after cocultivation, some edges of the leaf strips showed callus proliferation. After one month of selection, kanamycin-resistant calli were well formed. There were two types of calli—tight and hard calli, and friable and soft

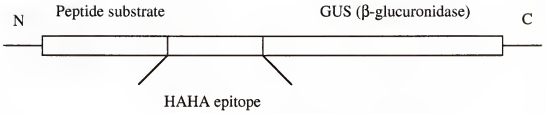


Figure 3-1. Diagram of structural organization of the fusion protein PHAG. The structure of the fusion protein PHAG is shown schematically. The drawing is not in actual scale.

calli. Both types of calli were examined for transgene expression using X-gluc staining.

Stable Expression of Phag in Tobacco Calli and suspension cells by GUS Staining

Expression of the transformed construct was confirmed by X-gluc staining for GUS expression. Kanamycin-resistant calli and suspension cells were stained blue with X-gluc indicating that the phag-construct was transformed into tobacco genome and stably expressed. Untransformed calli and suspension cells didn't show blue staining (data not shown).

Establishment of Transgenic Tobacco Suspension Cultures

One month after the leaf strip transformation, friable and soft calli were randomly selected and dropped into BY-2 medium for the initiation of the tobacco suspension culture. Hard calli were not selected because it is difficult to form a suspension culture from this type of calli. Tobacco cells grew faster in the culture medium than on agar. Three weeks later, a fast-growing and fine cell suspension culture formed. The suspension culture was maintained by weekly subculture. Periodic X-gluc staining confirmed that the foreign gene was stably integrated into the tobacco cell lines.

Expressed PHAG Can Be Immunoprecipitated and Immunoblotted by Anti-HA Monoclonal Antibody

Immunoprecipitation was performed with pBI121-phag transformed suspension cells and control pBI121 transformed cells. The immunoprecipitated

proteins of the test and control samples were assayed for GUS activity by fluorometric measurement (Table 3-1). Cell extract from untransformed tobacco

Table 3-1. GUS activities of tobacco cell extracts and immunoprecipitated proteins by anti-HA monoclonal antibodies

Source of sample	GUS activity (nM/min)
Untransformed cell extract	0.01
pBI121 transformed cell extract	4.5
pBI121-phag transformed cell extract	4.3
IP from pBI121 transformed cell extract	0.03
IP from pBI121-phag transformed cell extract	59.2

suspension cultures was used as control. GUS activity was detected in the immunoprecipitated proteins of pBI121-phag transformed cells, but not in vector pBI121 alone transformed cells. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-HA antibody. Figure 3-2 shows immunoblotted PHAG with predicted molecular weight of 76 kD in pBI121-phag transformed cell extract.

Phosphorylation of PHAG by Recombinant $CDPK_{\alpha}$

To investigate the phosphorylation status of the expressed pBI121-phag construct, phosphorylation assays of the immunoprecipitated proteins by recombinant $CDPK_{\alpha}$ were carried out. His_6-$CDPK_{\alpha}$ (Molecular Weight=58 kD) was used in the assay instead of GST-$CDPK_{\alpha}$ because the sizes of GST-$CDPK_{\alpha}$ (M. W. =83 kD) and PHAG-protein (M. W. =76 kD) are similar and they nearly

comigrated in the SDS-PAGE gel. Figure 3-3 shows that the PHAG-protein expressed in tobacco is phosphorylated by His_6-$CDPK_\alpha$ in the presence of calcium. The phosphorylation band in the X-ray film corresponds to the immunoblotted band in the immunoblot. The calcium-dependent phosphorylation of PHAG by $CDPK_\alpha$ is shown in Figure 3-4.

Phosphorylation of PHAG by Tobacco Cell Extract

Since the immunoprecipitated PHAG was phosphorylated by recombinant CDPK, whether PHAG-protein could be phosphorylated by the calcium-dependent protein kinase from plant cell extract was examined. For phosphorylation assay of PHAG by the tobacco extract, protein G agarose beads with immunoprecipitated proteins were suspended in the assay buffer and tobacco cell extract was added to the reaction mixture. Figure 3-5 shows that the plant extract phosphorylates immunoprecipitated PHAG in the presence of calcium, indicating strongly that plant CDPK will likely phosphorylate PHAG *in vivo*. Although PHAG was weakly phosphorylated by tobacco cell extract in the absence of calcium, the phosphorylation level of PHAG was stimulated in the presence of calcium. These results indicated that PHAG phosphorylation can be used as an indicator of the activation of CDPK in tobacco suspension cells.

Metabolic Labeling of PHAG by ^{32}P in Tobacco Suspension Cells and Change of Phosphorylation Status by Treatment with Hydrogen Peroxide

Tobacco suspension cells were labeled with $[^{32}P]H_3PO_4$ for an hour, then 2 mM H_2O_2 was added to cells and incubated for an additional hour. Control and

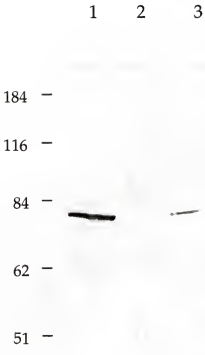
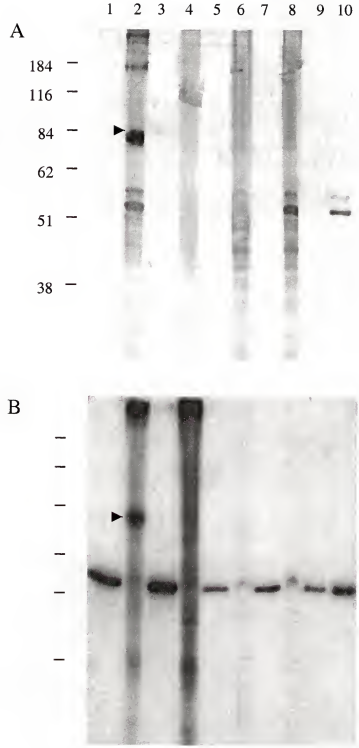


Figure 3-2. Expression of PHAG in transgenic tobacco cells and and immunodetection of PHAG. Cell extract from three-day-old IpBI121-phag transformed tobacco suspension cells was immunoprecipitated by anti-HA monoclonal antibody. Proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was overlaid with anti-HA antibody as described in Material and Methods. Lane 1 shows the immunoprecipitated PHAG with the predicted molecular weight of 76 kD. Lane 2 is control, immunoprecipitation was performed as in lane 1 except that control pBI121 transformed tobacco cell extract was used. Lane 3 is loaded with cell extract from pBI121-phag transformed tobacco cells. The positions of protein molecular weight markers are indicated at the left side of the figure. From top to bottom, their sizes are: 184, 116, 84, 62, and 51 kD.

Figure 3-3. Phosphorylation of immunoprecipitated PHAG by His_6-$CDPK_\alpha$. Immunoprecipitated proteins from transgenic tobacco suspension cell extracts were used in the phosphorylation reaction with His_6-$CDPK_\alpha$. Phosphorylation reactions were performed, and the reaction mixture was separated to supernatant and pellet as described in Material and Methods. Proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was overlaid with anti-HA antibody. The immunoblotted membrane was dried and exposed to an X-ray film. Panel A: immunoblot pattern of separated proteins. The arrow head indicates the position of PHAG. Panel B: autoradiograph of the immunoblotted membrane in panel A. The arrow head indicates the phosphorylation band corresponding to the immunoblotted band of PHAG in panel A. The band at molecular weight of 58 kD is from autophosphorylation of His_6-$CDPK_\alpha$. His-$CDPK_\alpha$ was present in supernatant fraction after separation of phosphorylation reaction mixture when Pansorbin cells were used for immunoprecipitation. In panel A and panel B: lanes 1-8 are from phosphorylation reactions with immunoprecipitated proteins and controls. Immunoprecipitated proteins for the reactions were prepared as below. In the immunoprecipitation for lanes 1 and 2, cell extract was mixed with antibody and then Pansorbin cells. The other three immunoprecipitations are controls: in the immunoprecipitation for lanes 3 and 4, only cell extract was mixed with Pansorbin cells; in the immunoprecipitation for lanes 5 and 6, only Pansorbin cells were used; in the immunoprecipitation for lanes 7 and 8, only antibody and Pansorbin cells were used. Lanes 1, 3, 5, and 7 are supernatants from each reaction. Lanes 2, 4, 6, and 8 are pellets from each reaction. Lane 9, His_6-$CDPK_\alpha$ alone control; lane 10, His_6-$CDPK_\alpha$ with antibody control. The positions of protein molecular weight markers are indicated at the left side of each panel. From top to bottom, they are: 184, 116, 84, 62, 51, and 38 kD.


A
1 2 3 4 5 6 7 8 9 10
184
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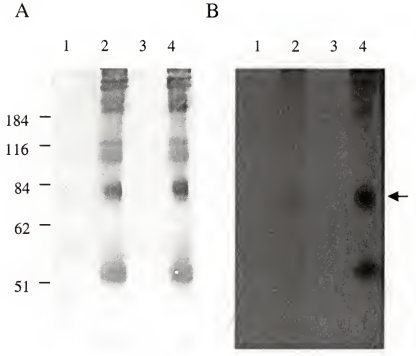

Figure 3-4. Phosphorylation of PHAG by His_6-$CDPK_\alpha$. Immunoprecipitated proteins from transgenic tobacco suspension cell extracts were used in the phosphorylation reaction with His_6-$CDPK_\alpha$ in the absence and presence of calcium. Phosphorylation reactions were performed, and the reaction mixture was separated to supernatant and pellet as described in Material and Methods. Proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was overlaid with anti-HA antibody. The immunoblotted membrane was dried and exposed to an X-ray film. Panel A: immunoblot pattern of separated proteins. The arrow indicates the position of PHAG in lanes 2 and 4. Panel B: autoradiograph of the immunoblotted membrane in panel A. The arrow indicates the phosphorylation band in lane 4 corresponding to the immunoblotted band of PHAG in panel A. The band at molecular weight of 58 kD is from autophosphorylation of His_6-$CDPK_\alpha$. His-$CDPK_\alpha$ was present in pellet fraction after separation of phosphorylation reaction mixture when protein G beads were used for immunoprecipitation. In panel A and panel B: phosphorylation reactions of immunoprecipitated proteins by His-$CDPK_\alpha$ were performed. In the reaction for lanes 1 and 2, calcium was absent in the reaction. In the reaction for lanes 3 and 4, calcium is present in the reaction. Lanes 1 and 3 are supernatants from each reaction. Lanes 2 and 4 are pellets from each reaction. The protein molecular weight markers used are the same as those in figure 3-3.

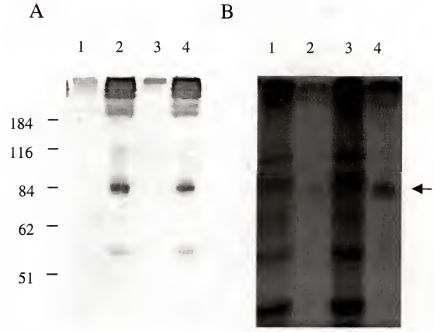

Figure 3-5. Phosphorylation of PHAG by tobacco cell extracts. Tobacco cell extract was immunoprecipitated with anti-HA monoclonal antibody. Protein Gagarose beads with immunoprecipitated proteins were used in the phosphorylation reaction with tobacco cell extract in the absence and presence of calcium. The reaction mixture was separated to supernatant and pellet as described in Material and Methods. Proteins in the reaction mixture was resolved by electrophoresis in a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was overlaid with anti-HA antibody. The immunoblotted membrane was dried and exposed to an X-ray film. Panel A: immunoblot pattern of separated proteins. The arrow indicates the position of PHAG in lanes 2 and 4. Panel B: autoradiograph of the immunoblotted membrane in panel A. The arrow indicates the position of the phosphorylated band in lanes 2 and 4 corresponding to the immunoblotted band of PHAG in panel A. In panel A and panel B: phosphorylation reactions of immunoprecipitated proteins by tobacco cell extracts were performed. In the reaction for lanes 1 and 2, calcium was absent in the reaction. In the reaction for lanes 3 and 4, calcium is present in the reaction. Lanes 1 and 3 are supernatants from each reaction. Lanes 2 and 4 are pellets from each reaction. The protein molecular weight markers used are the same as those in figure 3-3.

treated cells were homogenated and cell extracts were immunoprecipitated by anti-HA monoclonal antibody. Proteins were then resolved by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was exposed to an X-ray film after immunoblotting. Immunoblot and autoradiograph are shown in Figure 3-6. This experiment was repeated three times. Similar results were obtained. Even though the same amount of tobacco cells were used for the labeling, the amount of the immunostained PHAG in the lane loaded with H_2O_2 treated sample was more than that in the control lane. Quantitative analysis of immunoblots and autoradiographs were performed. There was 2-fold more PHAG present in H_2O_2 treated cells over control cells in the immunoblotting analysis, while H_2O_2 treatment caused a 5-fold increase of ^{32}P labeling of PHAG over control cells in the autoradiography analysis. These results show H_2O_2 treatment caused increased phosphorylation of PHAG in tobacco suspension cells.

Discussion

Design of the PHAG Construct

The peptide substrate obtained from random peptide phage display libraries was successfully expressed in transgenic tobacco cells as a GUS-fusion protein. The coding sequence for peptide substrate and double HA epitope tag was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and inserted into the binary vector plasmid pBI121. The resulting plasmid pBI121-phag was used for the transformation of tobacco (*Nicotiana*

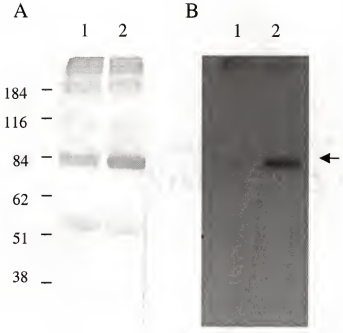

Figure 3-6. *In vivo* labeling of PHAG in transgenic tobacco suspension cells. Three-day-old transgenic tobacco cells were labeled with ^{32}P for one hour. Two mM H_2O_2 was then added to the labeling medium and the treatment lasted for an hour. For control, no H_2O_2 was added to the labeling medium. Labeled cells were washed and extracted. The same amount of cell extract from untreated and treated cells was used for the immunoprecipitation with anti-HA monoclonal antibody. Immunoprecipitated proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel, then electroblotted onto a nitrocellullose membrane. Immunoblot with anti-HA antibody was performed on the membrane. The membrane was dried and exposed to an X-ray film. Panel A: immunoblot pattern of separated proteins. The arrow indicates the position of PHAG. Panel B: autoradiograph of the immunoblotted membrane in panel A. The arrow indicates the position of the *in vivo* labeled band corresponding to the immunoblotted band of PHAG in panel A. In panel A and panel B: lane 1, immunoprecipitated proteins from tobacco cell extract without treatment; lane 2, immunoprecipitated proteins from tobacco cell extract treated with H_2O_2. The protein molecular weight markers used are the same as those in figure 3-3.

tabacum cv. Wisconsin 38) mediated by *Agrobacterium tumefaciens*. The expression of pBI121-phag construct was confirmed by GUS assays and immunoprecipitation of PHAG by anti-HA monoclonal antibody. The GUS fusion approach was chosen for the construction of the expression vector for peptide substrates based on previous reports. There was little direct expression of short foreign peptides, mainly due to the instability of peptides in bacterial (Derynck et al., 1984) and animal (Yamada et al., 1990) expression systems. This might also be true for foreign gene expression in plants. An antimicrobial peptide sarcotoxin IA was expressed successfully in transgenic tobacco plants by GUS fusion (Okamoto et al., 1998). The amounts of sacrotoxin IA in the sarcotoxin IA-GUS fusion protein transgenic plants were considerably higher than those of sacrotoxin IA in the single sarcotoxin IA peptide transgenic plants. It has been demonstrated that anti-HA antibody has very low background recognition for plant proteins (Sheen, 1996), so the HA epitope tag was selected and cloned into the expression vector for the detection and immunoprecipitation of the expressed foreign polypeptide.

Transformation and Expression of PHAG

Tobacco plants have been used for the transformation of pBI121-phag in the present study because many calcium requiring physiological processes have been observed in tobacco, and the manipulation of tobacco tissue and cell culture is feasible. The tobacco suspension cells have the advantage of perceiving external signals applied consistently and displaying uniform responses. Chandra et al. (1997) have demonstrated that the suspension cultured aequorin-transformed tobacco cells respond to external stimuli as do the intact transgenic

plants from which they were derived. In the present study, tobacco cell suspension culture was derived from the pBI121-phag transformed calli. Because several calli were used to initiate the cell culture, the cell population probably represents a random sample of cells with different sites of integration, copy numbers and transgene expression levels. Immunoblotting analysis using anti-HA antibody was performed with transgenic tobacco cell extract, a protein with the predicted molecular weight of 76 kD was detected in the transgenic cell extract.

Phosphorylation of PHAG

PHAG was immunoprecipitated by anti-HA monoclonal antibody from the transgenic tobacco cell extract. Immunoprecipitated PHAG was phosphorylated by His_6-$CDPK_\alpha$ and protein kinase in the tobacco cell extract in the presence of calcium, whereas in the metabolically labeled transgenic tobacco cells without any treatment, there was a low level of ^{32}P labeling corresponding to the immunoprecipitated PHAG band. This indicates that the expressed peptide substrate in the GUS-fusion form is phosphorylated at low level in tobacco cells under normal physiological conditions, but its phosphorylation is increased by elevated calcium. There was weak phosphorylation of PHAG by tobacco cell extract in the absence of calcium. This is consistent with the observed basal level phosphorylation of synthetic peptide substrate CSP by CDPK in the absence of calcium (see Chapter 2).

Hydrogen peroxide has been shown to stimulate an increase in cytosolic calcium (Levine et al., 1996; McAinsh et al., 1996; Price et al., 1994; Takahashi et al., 1998). Reactive oxygen species are produced rapidly after elicitor treatment

or infection with an avirulent pathogen strain. Hydrogen peroxide produced by challenged plants/cells directly reduces pathogen viability. Hydrogen peroxide mediates the cross-linking of cell wall structural proteins (Bradley et al., 1992). Hydrogen peroxide also functions as a local trigger of programmed cell death in challenged cells and as a diffusible signal for the induction of genes encoding cellular protectants such as glutathion S-transferase and glutathione peroxidase (Levine et al., 1996; Levine et al., 1994). In the present study, the enhanced ^{32}P labeling of PHAG upon H_2O_2 treatment indicates an increased phosphorylation of PHAG by a tobacco calcium-dependent protein kinase, most likely a CDPK. It is not known which kinase is involved, nor the identity of the endogenous substrate. An *in vitro* substrate of CDPK, serine acetyltransferase (SAT), was found by interaction cloning (B.-C. Yoo and A. C. Harmon, personal communication). SAT is phosphorylated by CDPK *in vitro*, and the phosphorylation of SAT relieves the feedback inhibition of SAT by cysteine that can be used for the synthesis of glutathione. Glutathione was reported to regulate defense-related gene expression as well (Wingate et al., 1988). It is possible that H_2O_2 causes increased $[Ca^{2+}]$ that activates CDPK, this activated CDPK then phosphorylates SAT that leads to greater cysteine production that can be used for the synthesis of glutathione.

A transgenic tobacco cell suspension culture was established in this study. The peptide substrate obtained from the screening of random peptide phage display libraries was expressed in the transgenic cells in the GUS-fusion form. Hydrogen peroxide treatment of the transgenic tobacco cells caused increased phosphorylation of the CDPK peptide substrate in *vivo*. Specific antibodies against the phosphorylated and unphosphorylated peptide substrate are desired

to facilitate future studies. A number of physiological processes involving calcium can be investigated then for the involvement of calcium-dependent protein kinase using the established transgenic tobacco cell culture.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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